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Resistance to macrocyclic lactones in the parasitic mite *Psoroptes ovis* (Acari: Psoroptidae)

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A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Veterinary Parasitology MSc(R) in the Faculty of Science, School of Biological Sciences.

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ABSTRACT

Psoroptes ovis (Hering, 1858) is an astigmatid mite that causes the highly contagious and debilitating disease known as sheep scab. This ectoparasitic infection has high economic and welfare costs for British sheep farming.

The condition is augmented through a host hyper-immune response that instigates the production of an exudate, antigenic serum containing immunoglobulins in response to the mite recombinant protein, Pso o 2. This reaction causes intense pruritus and epidermal inflammation, which rapidly leads to severe deterioration in host condition. The most commonly used therapeutic treatment is topically or intramuscularly administered macrocylic lactones (MLs). Over the past decade there have been widespread reports of scab outbreaks that do not appear to respond to this group of compounds. A study by Doherty *et al.* (2018) was the first to demonstrate evidence of *P. ovis* resistance to the ML, moxidectin, in non-responding populations, through a formulated bioassay. The data described in the present study supports the preceding evidence of moxidectin resistance in these non-responding populations, whilst also indicating that there are varying levels of intraspecific resistance to all three commonly used ML treatment compounds: ivermectin, doramectin and moxidectin. Outbreak samples that did not show evidence of resistance (despite being secondary outbreaks) and inviable samples received due to storage or transport method, highlights the widespread insufficient knowledge and understanding of *P. ovis,* its control and management, within the agricultural community. This further demonstrates the importance of strong biosecurity and stringent monitoring of regional flock health, necessary to maintain sustainable low disease prevalence within the United Kingdom.

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AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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CHAPTER 1

INTRODUCTION

1.1 THE BIOLOGY OF PSOROPTES OVIS AND CLINICAL INFECTION

1.1.1 Classification and taxonomy

The non-burrowing arthropod mite, *Psoroptes ovis* (Hering, 1838), is an obligate ectoparasite belonging to the family Psoroptidae (Order Astigmata). *Psoroptes* mites are seen in all seasons and can infect a wide variety of mammalian hosts including cattle, rabbits, various camelids and sheep (Shilston, 1915; Downing, 1936). *P. ovis* are traditionally given different species names depending on the primary host species they infect (Wall and Kolbe, 2005). However, molecular studies comparing and contrasting the second mitochondrial internal transcribed spacer (ITS-2) of different *P. ovis* variants, reveal no genetic differentiation between *Psoroptes* mites that infect different host species (Pegler *et al.,* 2005). Despite this, there are distinct phenotypic differences between different host-derived *P. ovis* and pathological differences in host responses to infection. *Psoroptes* infection can be separated into two types: infection resulting in bodily mange and infection resulting in acute otoacariasis (Bates, 1999). It has been suggested that ear forms may represent the ancestral interaction between mites and wild host with the more pathogenic body-form of infection being associated with more susceptible domesticated sheep (Pegler *et al*., 2005).

Despite the variations in nomenclature, the name *Psoroptes ovis* is regarded as having taxonomic priority within the genus, as this was the nominal designation given when first described by Hering (Hering, 1838; Wall and Kolbe, 2005). The mite is the causative agent of Psoroptic acariasis or mange- the clinical disease colloquially known as 'Sheep Scab' (Shilston, 1915; Tarry, 1974; Lewis, 1997; Bates, 1999). Sheep scab is considered to be the most important ovine ectoparasite infection in Great Britain and many other countries, due to the significant economic

and welfare impacts to livestock and agriculture (Kirkwood and Quick, 1982; O'Brien, 1999; van den Broek and Huntley, 2003).

Records of the disease date back to pre-180BC, in the writings of classical authors such as Cato and Virgil alongside biblical parallels within the Old Testament (Kirkwood, 1986), however, the causal agent was not identified until recognised as a mite by Walz in 1809.

In the United Kingdom, Sheep Scab has been of high national importance, predominantly due to its economical and welfare impacts, seen throughout the period of British Agricultural Revolutions. Changes in control strategy and nationwide policy during the 19th century, particularly with the deregulation of dipping, have resulted in a higher disease prevalence across the British Isles (Nieuwhof and Bishop, 2005).

1.1.2 Morphology and Life History

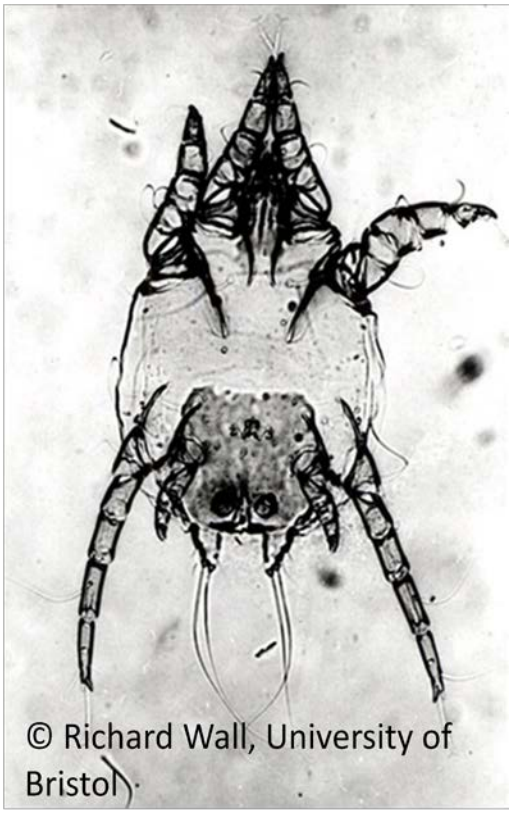


Fig. 1.1 Microscopic capture of Male *Psoroptes ovis* mite.

The characteristic distinguishing features of *Psoroptes ovis* are its pointed mouthparts and three-jointed pretarsi bearing pulvilli (Wright and Deloach, 1980). The body profile is oval and legs protrude from the body margin. Adult females have jointed pre-tarsi on the first, second and fourth pairs of legs with long setae on the third. Conversely, adult males (Fig. 1.1) are smaller and distinguished by coupled posterior lobes and posterior copulatory suckers (Sanders *et al.,* 2000). The part of the body incorporating the feeding organs is called the gnathosoma and the posterior part of the body is the opisthosoma.

The lengths of the posterior opisthosomal setae of adult males were believed to be the distinguishing feature for *P. ovis* host-derived species (Sweatman, 1958), however these measurements may vary over the course of infection, with setae length inversely correlated to infection lesion age and so making this character taxonomically unreliable (Wright *et al.,* 1984).

The early observations of the parasite's life-history were undertaken by Gerlach in 1857 and debate over the pattern and rate of development continued throughout the early literature with highly divergent observations (Shilston, 1915). In-depth analysis of these early population observations and assessment of the *P. ovis* life-cycle were undertaken in South Africa in the early 20th century using confined populations on living sheep (Downing, 1936). This showed that *P. ovis* undergoes five life-cycle stages as follows: egg, hexapod larva, two octopod nymphal stages, (designated as the protonymph and tritonymph) and the octopod adult.

At the larval stage, both sexes have 3 pairs of legs and moult in to protonymphs after 2 days. Female nymphs can be distinguished from males by size and the presence of dorsoposterior tubercles, however, distinguishing between nymphal stages depends on the number of metapodosomal setae and location of cuticular pits (Sanders *et al.,* 2000). In astigmatid mite species, the protonymph moults directly into the tritonymph, omitting the deutonymphal instar seen in other mite species e.g- the spider mite, *Tetranychus urticae* (Gotoh, 1986; Sanders *et al.,* 2000). Female adults are substantially larger than males (Sweatman, 1958). Males develop posterior copulatory tubercles (Fig. 1.1). Mature males form pairings with

female tritonymphs and copulation occurs when the attached nymph moults into the adult stage (Shilston, 1916; Downing, 1936).

All developmental stages occur on the host and key morphological features distinguish each stage of the life-cycle, which can be completed in approximately 10-19 days, with each life cycle stage taking at least 2 days (Downing, 1936; Sweatman, 1958; Sanders *et al.,* 2000). However, the exact rates of development are highly dependent on temperature and humidity. For example, Shilston (1915) observed that there is a period of two to nine days between egg oviposition and hatching, dependent on the egg's proximity to the host epidermis. Female mites can live for 11-42 days, depending on the conditions of temperature, humidity and food availability. In a favourable environment, females can deposit up to 90 eggs during one life-cycle (Stockman and Berry, 1913).

Environmental transmission may be important in the epidemiology of sheep scab, so off host survival is an important factor. As mentioned earlier, studies have shown that off-host survival is highly dependent on temperature and humidity, therefore survival may be extremely variable (Hertwig, 1835; Salmon and Stiles, 1903; Bedford, 1915; Dill, 1920; Tarry, 1974; Kirkwood, 1986). Mites can remain infectious off host for a maximum of 15 days (O'Brien *et al.,* 1994).

Although outbreaks of *P. ovis* can be seen year-round in the United Kingdom, it has been observed consistently that infection incidence peaks during the winter months (Shilston, 1915; Downing, 1936). A Leslie matrix model was used to predict that a population of *Psoroptes* mites on a sheep is likely to grow at a rate of about 11% daily with the population doubling every 6.3 days. Mite populations increase exponentially after an initial period of instability once infections have established (Wall *et al.,* 1999). Hence, *P. ovis* infection can lead to rapid disease progression.

1.1.3 Pathology

Although it is a non-burrowing mite, *P. ovis* causes chronic dermatitis and severe pruritus. Scanning electron microscopy shows that *Psoroptes* have mouthparts adapted for epidermal abrasion and liquid feeding (Mathieson and Lehane, 2002),

but not, as was originally thought, for piercing through the epidermis (Downing, 1936). *Psoroptes* mouthparts do not appear to penetrate beyond the outermost layer of the host epidermis, the stratum corneum (Sinclair and Kirkwood, 1983). Mites feed superficially on a liquid emulsion of lymph and bacteria produced at the host epidermis layer (Rafferty and Gray, 1987; Synge *et al*., 1995) ingested through their pharynx and oesophagus. The pharynx is lined with a cuticle heavily composed of sclerotin and accompanied by three dilator muscles thought to draw liquid through the roof of the gnathosoma. The structure of the foregut is similar to other species of Acari except for a pharyngo-oesophageal valve which is not a general feature of most Acari, however is found in some related astigmatid mites such as the house dust mite, *Dermatophagoides farinae* (Brody *et al.,* 1972).

Infestation may be localised and often subclinical, particularly in the early stages of infection. In localised infection mites commonly aggregate on the flanks, back or legs of the host (Sargison, 1995; Bates, 1997). In the field, natural subclinical infections can endure for 2-8 months, followed by an exponential population growth phase in which clinical signs advance (Sargison, 1995; Bates, 1997). When infection is generalised, mites may extend across the entire body.

The clinical pathology of a *P. ovis* infection is the result of a hyper-immune response by the host. When the mites establish themselves on the host, guanine-rich faecal matter and antigenic salivary material is deposited on the skin, causing an immediate immune reaction. Homologues of the house dust mite allergen proteins have been generated from *P. ovis* cDNA, which react with immunoglobulin G and other serum antibodies to elicit the epidermal inflammatory effects in sheep (Bates, 1997; Lewis, 1997).

Microflora in the parasite midgut and antigenic material from bacterial digestion may influence the severity of host-response. The behavioural reactions of sheep to the infection arise from irritation and inflammation. Rubbing causes skin abrasion and epidermal tears allowing for subcutaneous microbial infiltration and secondary infection. *Serratia marcescens* has been linked with mite infection and is known to cause disease and septicaemia in mammals (Mathieson and Lehane, 1996). Increases in *P. ovis* specific IgG and IgE have been detected in sheep at 2-3 and 4-5

weeks after an initial infection. Subsequently, infection then results in rapid anamnestic IgE production, but not IgG. Pathology and immune-response progression may vary between sheep breeds (Fisher *et al.,* 1998; Smith *et al.,* 2001).

The disease is highly contagious with transmission resulting from direct contact between animals, or indirectly from handling equipment or shared environmental components. Due to the high rate of population growth, infection with small numbers of ovigerous female *P. ovis*, can lead to a full clinical infestation in 6 to 8 weeks (Babcock and Black, 1933), although progression of the clinical disease varies on whether or not it is a primary infection or whether the sheep has been infected beforehand. In primary infections there is an initial "lag" phase followed by a fast-expanding "growth" phase with an increase in abundance and size of scab lesions. Secondary infection growth has a prolonged lag phase and a less aggressive growth phase, as a result of the immune system protection of the previously infected sheep (Bates, 2000).

1.1.4 Clinical signs and diagnosis

Clinical signs of sheep scab vary over the course of an infection and are dependent upon environmental factors. Typical signs include rapid weight loss through preoccupation with, and agitation of, clinical symptoms coupled with protein loss through exuding immunogenic serum. Other responsive signs include tongue protrusion, lip smacking, rubbing and nibbling at the fleece; which can result in hair loss, epidermal damage, epileptiform fits and self-trauma (Fig. 1.2). Following this, possible fatalities arise from secondary infections, pneumonia and severe dehydration (Tarry, 1974; Kirkwood, 1980; Bates, 1997; Bygrave *et al.,* 1993).

Lesions and cutaneous inflammation are generated with progressing dermatitis and self-trauma reveals ulcers and epidermal abrasion. Lesions extend to aforementioned areas of common mite localisation and an antigenic serum exudate is produced alongside pustule discharge, staining the wool yellow as the exudate coagulates (Downing, 1936; Spence, 1949; Sargison, 1995; Bates, 1997).

Fig. 1.2. Veterinarian taking scrape from *Psoroptes ovis* mite infected area on an ewe (Wiltshire, 2018, © C. Sturgess-Osborne).

Sargison *et al.* (2005) has shown that when a pregnant ewe contracts sheep scab, a loss of condition is positively correlated with scab severity. Lambs of severely infected ewes were seen to have a lower birth weight than those mildly infected or free from *Psoroptes*. Pregnancy also causes other complications during scab infection. The pharmacokinetic parameters of treatments can change during pregnancy. A substantial decrease in the half-life of moxidectin elimination was found in pregnant ewes, suggesting that treatment longevity is reduced during pregnancy (Pérez *et al.,* 2014).

In bovine infection, *P. ovis (syn P. bovis)* extends fully across a lesion, whereas in sheep they aggregate at the edge between the lesion and normal skin. This is likely due to the uninhabitable local environment created by the fast-drying serum exudates at the lesion on sheep, which is not found in cattle infection. In cattle scab, lesions remain moist, creating an optimal humidity for mites (Kirkwood, 1986).

1.1.5 Epidemiology and distribution

Sheep scab epidemiology in 21st century Britain is not uniform, with higher concentrations of distribution found in Scotland, Northern England and Wales (Bisdorff *et al.,* 2006). A more recent control survey found that 60% of the sheep

farming community did not use prophylaxis treatment. This is suspected to be on economic and efficiency grounds and due to confusion within the farming community on effective treatment strategy, as well as avoiding treatment interruption of seasonal husbandry practices. Application of acaricidal compounds during the summer period is convenient in relation to other on-farm activities such as shearing, prevention of other treatment-susceptible livestock ectoparasites and treating around the prenatal periods that prevent dipping in winter. The ineffective treatment timing in respect to sheep scab seasonality can result in the re-emergence of scab, lowering confidence in prophylaxis within the British farming community (Bisdorff and Wall, 2008).

It is considered that upland and lowland areas in Britain need discrete control systems, as differences in husbandry practice and environmental factors lend to distinct variations in transmission risks and disease spread between flocks (Nixon *et al.,* 2017). Observations across the literature clearly demonstrate that population dynamics and epidemiology are influenced by a multitude of interacting factors, both intrinsic within the microclimate of the host-parasite interaction coupled with external environmental contributions (Bates, 1997; O'Brien, 1999). The complexity of these dynamic systems causes the ongoing difficulty in effective control of the disease.

1.1.6 Economic and veterinary importance

There are approximately 35 million agricultural sheep in the UK, equating to £1,197 million towards agricultural-based income in meat production alone (UK Government Statistics, 2017). A calculated estimate for the annual cost of *P. ovis* to the UK was £8.3 million (Nieuwhof and Bishop, 2005). However, this calculation did not take into account the monetary losses from labour, transmission impacts- such as movement restrictions limiting land use and subclinical disease impacting productivity in sheep and other susceptible mammals. Therefore, the cost is likely a lot higher, even before incorporating an economic inflation adjustment for the current year (Nixon *et al.,* 2017). Production loss is a large component around the

significant economic impacts of *P. ovis*. Psoroptic mange and other prevalent ovine ectoparasite diseases, such as cutaneous myiasis, have high welfare impacts and risk of mortality on sheep of all ages (Scott *et al.,* 2007).

1.2 THE HISTORY AND CONTROL OF SHEEP SCAB IN GREAT BRITAIN

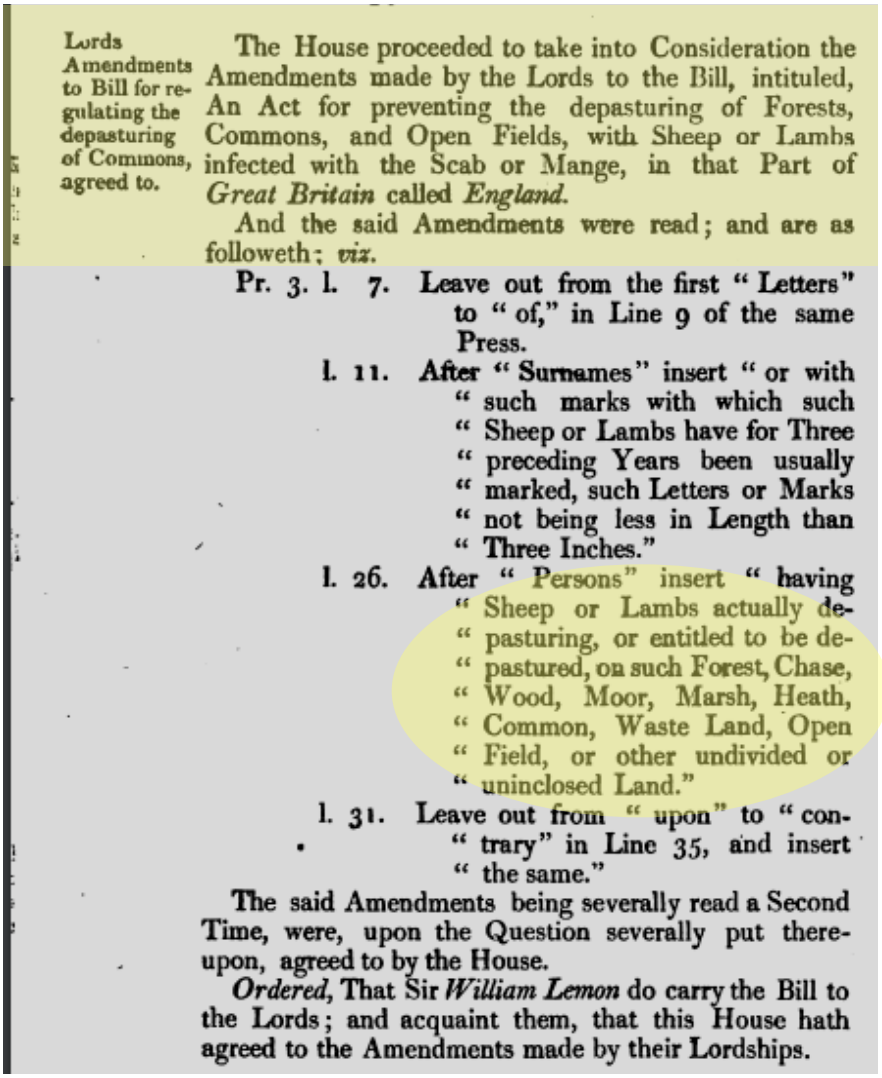
1.2.1 History of psoroptic mange control

Records of scab prevention date back as far as 180 BC. Cato the Elder advised that after shearing, sheep should be treated with a mixture of strained olive oil lees, water that had soaked ("steeped") plants of the genus *Lupinus* and dregs of barrelled wine. His writings then instruct that one should let the sheep "sweat profusely for 2-3 days" followed by dipping in saltwater, which he claimed would prevent future scab and tick infection (Cato, 180BC). Similar treatment methods using lard and mercury were used during the middle-ages. A virulent outbreak of scab resulting major economic losses was recorded in Belgium (then Flanders) in 1275 (Stephenson, 1988). This outbreak was believed to have been imported from Britain, due to the country's reliance on imported English sheep wool. In the UK, confirmed records of scab were recorded in 1807, when there were 2573 outbreaks, rising to 3536 in 1895 (Kirkwood, 1986).

It is thought that the earliest legislation imposed to control sheep scab in Britain dates from AD 949, when King Hywell Dda of Wales forbade trade of infected flocks between November and April (Kirkwood, 1986).The first official British law regulating scab control was a House of Lords amendment passed in 1798, preventing the grazing of animals on pastures that harboured scab infected sheep (Journals of the House of Commons, 1798; Fig. 1.3).

The first commercially available dips in Great Britain, based on arsenic, became available in the mid-19th century. After a period of experimenting between 1843-1852 with arsenic and sulphur, William Cooper formulated 'Cooper's Dip' (Downing, 1936). In the years previous to this, scab was inadequately managed with treatments containing soot, goose-grease, tobacco and flour of brimstone (Ellis,

1749). Various other compounds were trialled over the 19th century using tar, rotenone and creosote.



Lords Amendments to Bill for regulating the depasturing of Commons, agreed to.

The House proceeded to take into Consideration the Amendments made by the Lords to the Bill, intituled, An Act for preventing the depasturing of Forests, Commons, and Open Fields, with Sheep or Lambs infected with the Scab or Mange, in that Part of *Great Britain* called *England*.

And the said Amendments were read; and are as followeth; *viz.*

Pr. 3. l. 7. Leave out from the first "Letters" to "of," in Line 9 of the same Press.

l. 11. After "Surnames" insert "or with "such marks with which such "Sheep or Lambs have for Three "preceding Years been usually "marked, such Letters or Marks "not being less in Length than "Three Inches."

l. 26. After "Persons" insert "having "Sheep or Lambs actually de- "pasturing, or entitled to be de- "pastured, on such Forest, Chase, "Wood, Moor, Marsh, Heath, "Common, Waste Land, Open "Field, or other undivided or "uninclosed Land."

l. 31. Leave out from "upon" to "con- "trary" in Line 35, and insert "the same."

The said Amendments being severally read a Second Time, were, upon the Question severally put thereupon, agreed to by the House.

Ordered, That Sir *William Lemon* do carry the Bill to the Lords; and acquaint them, that this House hath agreed to the Amendments made by their Lordships.

Fig. 1.3. Journals of the House of Commons agreement to Lords Amendment on the depasturing act in England 1798 (adapted for clarity).

Initial statutory control was achieved through the Sheep Scab Order of 1898, which required authorities to liaise with veterinary surgeons for diagnosis. Case prevalence dropped from 2514 in 1898 to 1379 by 1901, however the extent of which this was due to the early Scab Order has been debated (Page, 1969). Compulsory single dipping was introduced in 1905 and a second Scab Order was imposed in 1907 (Board of Agriculture and Fisheries, 1906; Downing, 1936)

regulating the use of a new dip formulation with lime sulphur, arsenic and phenols (Salmon and Stiles, 1900; Kirkwood, 1985).

Double dipping was introduced during the early 20th Century, 1914. In 1920, it was understood that no available single dip ensured egg mortality, thus a second dip was needed to ensure that newly hatched mites were killed. Local authorities were also given the power to impose single, double or triple annual dipping in their specific regions. A 1928 act limited treatment use to ministry approved compounds only, as well as demanding euthanasia of infected individuals and enforcing movement and isolation restrictions on infected flocks (Downing, 1936; Spence, 1951).

Pre-1947 treatments had low persistence until the development of organochlorine compounds which gave high levels of mortality and residual protection for up to 3 months (O'Brien, 1999). In 1948, the introduction of gamma-hexachlorocyclohexane (GHCH [*syn* lindane or γ-HCCH]) and compulsory dipping eventually resulted in the eradication of Sheep Scab in 1952. A re-emergence occurred in 1972, which is believed to be the result of import and initial infection misidentification during the transport of infected sheep from Ireland. The outbreak was eventually identified on January 1st, 1973 in Lancashire, by which time considerable dispersal had already occurred from the initial point of introduction (Kirkwood, 1985).

In 1972 lindane (Gamma H-CH) was the only evidence-backed treatment available (Tarry, 1974). Environmental and health concerns, plus evidence of scab mites developing resistance to lindane (Rosa and Lukovich, 1962), led to the introduction and use of a generation of less persistent synthetic pyrethroids (high cis-cypermethrin and flumethrin) and organophosphate, with diazinon and propetamphos being licensed for Sheep Scab in 1981 (Tarry, 1974; Bates, 2004). Propetamphos was considered to be a particularly effective compound as it was also administered for blowfly and lice management (Kirkwood and Quick, 1981; Kirkwood and Quick, 1982).

Dip-formulae containing lindane and other organochlorines were voluntarily withdrawn in 1984 primarily due to risks of residual meat contamination although a

wide range of environmental issues are now known to result from their use (Henderson, D., 1991; Kunz and Kemp, 1994). Synthetic pyrethroid (SPs) insecticides were banned in the 1990s because of environmental concerns.

Currently, only two classes of acaricidal compound are still licensed for treatment of *P. ovis*: diazinon organophosphate dip (OPs), and injectable macrocyclic lactones (MLs) (Lewis, 1997). OP insecticides have phosphate radicals which act as carboxylic esterase inhibitors through phosphorylating enzymes at the active site. Toxicological impacts of OPs are driven by the inhibition of acetylcholinesterase within the nervous system. Case studies throughout the 1970s showed that the resulting excess of acetyl-choline, at cholinergic synapses, disabled somatic nerve transmission, sparing only nerves of the adrenergic nervous system (synapses of the neurotransmitters epinephrine and noradrenaline) (Ahlquist, 1948; Namba *et al.,* 1971). Considerable concerns have been raised over the human health impacts of exposure to OPs. A study showed that the OP insecticide used in scab treatment, diazinon, acted as an anticholinesterase in healthy human erythrocytes (Namba *et al.,* 1971). This mechanism was not responsible for the neurotoxicity of OPs seen in humans. Neuropathic abnormalities were thought to be a result of esterase, over cholinesterase, inhibition from the OP containing compound- triorthocresyl phosphate (Cavanagh *et al.,* 1961). Additional studies of the mechanisms behind the interaction of OP compounds with the neurodevelopmental gene pathways and cell apoptosis, may reveal the mechanism of action behind correlated user neurotoxicity (Slotkin and Seidler, 2012). Some laboratory studies suggest OPs cause changes of expression in lipid-based enzymatic pathways, serine hydrolases and cAMP expression. Several systems in the brain are modulated by lipases sensitive to oraganophosphate exposure. For example, monoacylglycerol lipase and the fatty acid amide hydrolase, regulate neural endocannabinoid levels (a group of endogenous neurotransmitters involved in mobility, memory and pain mechanisms). OP exposure has been found to inhibit these enzymes in mice, resulting in augmented endocannabinoid concentration in the brain (Casida *et al.,* 2008). Neurotrophic abnormalities such as defective axonal transport and

alterations in the intensity and timing of neurotransmitter signalling, lead to behavioural and physiological defects in people working with OPs (Slotkin, 2004).

MLs now available for scab management and prophylaxis include the avermectins (ivermectin and doramectin) and the milbemycin, moxidectin. They are the highest selling anthelmintics globally. MLs are endectocides and can kill both endo- and ectoparasites (Shoop *et al.,* 1995). They are also used for nematode control in horticulture and the control of river blindness (onchocerciasis) in humans (Omura and Crump, 2004). These chemically related compounds are derived from fermentation products of Actinomycetales bacteria, *Streptomyces*. Ivermectin and doramectin are derived from the bacterium *Streptomyces avermitilis*; moxidectin was originally derived from *S. hygroscopicus* and then from *S. cyaneogriseus* from 1983 (Chiu *et al.,* 1987*)*. Within nematode and arthropod nervous systems, the primary mode of action behind all MLs is to modulate the action of chloride ion channels. All three of the compounds used for sheep scab treatment are 16-membered lactone compounds, with potent insecticidal and anthelmintic properties. The milbemycin, moxidectin, differs from the other avermectins as it is unglycosylated and protonated, thus unsubstituted and lacking a bisoleandrosyloxy (disaccharide) substituent at C_{13} (Campbell *et al.,* 1983). Doramectin is a synthetically-modified avermectin, with a cyclohexyl, $C_6H_{11,}$ group at C_{25} and having a greater active period and plasma half-life than ivermectin (Shoop *et al.,* 1994; Banks *et al.,* 2000; Voyvoda *et al.,* 2005). Homogenous structural components allow for all to bind glutamate-gated chloride channel receptors (Burg *et al.,* 1979; Egerton *et al.,* 1979; Campbell *et al.,* 1983; Campbell, 1989; Prichard *et al.,* 2012; Fig.1.4).

ML pharmacophores have a low affinity for mammalian ligand-gated chloride channels and glutamate-gated chloride channels are only found in protostome invertebrates, thus MLs do not readily cross the blood-brain barrier of mammals. Therefore, the use of these compounds is considered to have a high margin of mammalian safety and they do not have negative effects in the mammalian peripheral nervous systems at standard dosage concentrations (Slimko *et al.,* 2002;

Wolstenholme, 2012). The MLs act as substrates for permeability glycoproteins (P-gp), being ATP-dependent efflux membrane pumps.

Ivermectin was the first ML developed for use in veterinary treatment. Its vermicidal effects are exerted through selectively binding glutamate-gated chloride channels in invertebrates. In the compound class, other ML compounds can cause the potentiation of bind/release mechanisms of the neurotransmitter, gamma-aminobutyric acid (GABA). The increase in permeability of chloride ions when binding to glutamate-gated Cl^- channels causes an influx of chloride ions resulting in hyperpolarisation of nerve cells and consequential paralysis and death of the parasite- as schematically represented in Appendix A (Egerton *et al.,* 1979; Campbell *et al.,* 1984**;** Cully *et al* 1996; Slimko *et al.,* 2002; Lanusse *et al.,* 2009) .

In 1983, scab incidence was very low at between 10 and 100 outbreaks per year (Page, 1969; Loxam, 1974; Kirkwood, 1986). However, the last remaining cases of scab could not be eradicated; it is thought that this was due in part to the lower residual activity of OPs and SPs, though farmer non-compliance may also have been important. Given that the vast majority of sheep dipped each year in the UK did not have scab and that dipping was both expensive and environmentally damaging, the government decided to abandon attempts at national eradication; scab was deregulated in 1992 and national compulsory dipping was abandoned. Following this, the number of scab outbreaks rose dramatically and there are now thought to be approximately 5,000-10,000 outbreaks each year (Bisdorff *et al.,* 2006; Nixon *et al.,* 2017).

One of the economic factors contributing to the high cost of *P. ovis* is the economic loss in animal product-based food sales. Meat withdrawal is due to the presence of treatment residues in blood plasma after treatment. The plasma of cattle injected with moxidectin was assessed against the plasma concentration in their calves which had not been treated. Blood was taken from cows and their calves at intervals over 120 days and centrifuged for analysis by chromatography. It was found that circa 5% of the moxidectin, which had been administered at standard dosage recommendations, had been transferred to the calf via the mother's milk (Alvierie *et al.,* 1996). The meat withdrawal periods of the ML products used in the

current study are as follows: subcutaneous injection of 2% Long-Action Cydectin (moxidectin) is administered at the base of the sheep ear and meat withdrawal period after treatment is 104 days. For Dectomax 10mg/ml (doramectin) which is administered as an intramuscular injection, the withdrawal is 70 days, and this is the only licensed treatment that treats scab in a single injection. Dectomax is also a popular treatment in the farming community because it treats gastrointestinal worms and lung worm alongside scab in sheep and cattle. Alternatively, Oramec (ivermectin) is given via oral drench and meat withdrawal is only 6 days. However, neither ivermectin nor doramectin provide any long-term length of protection, whilst Cydectin (moxidectin) gives 60 days residual protection against *P. ovis* re-infection (*pers comm.* Zoetis, product technical team, 2017).

Ivermectin is highly life-stage specific and does not target as wide a range of species as the other avermectins (Campbell and Benz, 1984). These differences in the treatment are considered when farmers and veterinarians are making decisions on treating a new scab outbreak. OP dips are the most economical control method for sheep scab. Despite this, a survey of 966 farmers located across the UK found that the majority, an average of 56.3%, used injectable insecticides to treat scab whilst only 27.5% used a dip and 16.3% combined both (Bisdorff and Wall, 2008). While the injectable MLs (ivermectin, doramectin and moxidectin) and the full-immersion plunge-dipping compound, diazinon, are the only compounds licensed for the treatment of sheep scab, a recent survey in Wales showed that there is considerable confusion in the use of appropriate products with numerous cases observed in which farmers used inappropriate products in an attempt to treat scab (Chivers *et al.,* 2018).

1.3 DEVELOPMENT OF RESISTANCE

1.3.1 Records of resistant *Psoroptes ovis*

Where the mass-scale administration of any compounds is used for parasite control, particularly where there is limited variation or rotation in the treatments available, selection for resistance is expected to be almost inevitable (Bates, 1998).

Accordingly, drug-resistance in a wide range of ovine parasites has been reported (Synge *et al.,* 1995). The first report of pyrethroid-resistant scab was in 1995. Resistance may have been facilitated by asymptomatic scab cases being exposed to low dose concentrations when pyrethroids were used to treat other ectoparasites. It was predicted that resistance of ovine parasites to avermectins and milbemycins could develop due to the incomplete treatment and low-level exposure in subclinical scab populations (Coles, 1998). The use of MLs to manage scab might also facilitate the section for resistance in nematode parasites. Resistance in ovine parasites has been an increasingly common observation over the past two decades, for example with resistance seen in the nematode *Teladosagia circumcincta* to benzimidazoles, tetrahydropyrimidines, imidazothiazoles and macrocyclic lactones (Sargison *et al.,* 2003).

1.3.2 Recent emergence of persistent scab outbreaks

A recent study identified resistance to moxidectin in British sheep flocks (Doherty *et al.,* 2018). Failure of ML treatment for *Psoroptes* is not only limited to sheep scab outbreaks. A trial of injectable ivermectin to treat *Psoroptes* in Belgian blue cattle (described as *Psoroptes bovis*), failed to cure over 50% of hosts (Lekimme *et al.,* 2010) leading to a presumption that the mites were resistant. Nevertheless, other studies have shown differences in efficacy between various products containing ivermectin (Genchi *et al.,* 2008) and the trial by Lekimme *et al.,* (2010) was of limited size and did not have appropriate controls.

The study conducted by Doherty *et al.* (2018) was carried out after consistent reports of outbreaks that were non-responsive to treatment in areas of high sheep scab prevalence across Britain. The results demonstrated that there are outbreaks of *P. ovis* that are resistant to moxidectin within the UK. The authors suggested that given the similar pharmacological interactions between the different MLs and substrates at chloride channels, these resistance populations were likely to show tolerance to other established ML treatments within this class, e.g. ivermectin and doramectin based compounds. As described (See Chapter 1.2.1), MLs act as

substrates for permeability glycoproteins (P-gp), being ATP-dependent efflux membrane pumps at chloride channel receptors, shown schematically in Appendix A (Fig. 1.5).

All three MLs currently used to treat sheep scab (class avermectin: ivermectin and doramectin and class milbemycin: moxidectin) have superimposable structural ML groups (Shoop *et al.* 1995; Fig. 1.4). It has been observed that ivermectin-resistant worms present lower susceptibility to moxidectin treatment (Conder *et al,.* 1993). Further study on this phenomenon showed that *Haemonchus contortus* selected for moxidectin resistance, also presented lower susceptibility to ivermectin treatment (Molento *et al.,* 1999). Whilst the mechanism of resistance in sheep scab is not yet fully understood, the results of the (2018) Doherty *et al.* study showing *P. ovis* resistance to moxidectin in the UK, commands investigation into cross resistance to the other MLs in non-responding *P. ovis* outbreak populations.

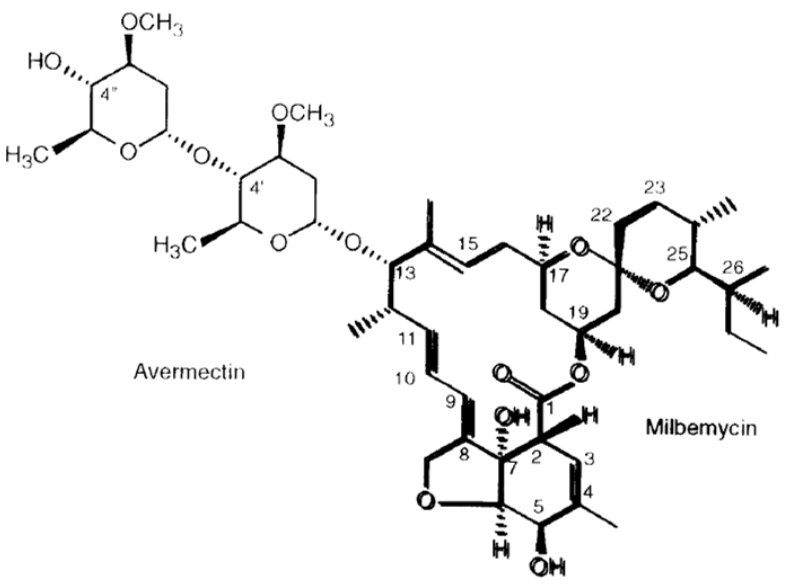


Fig. 1.4. Superimposition of avermectin and milbemycin molecules (from Shoop *et al.,* 1995).

By determining a shared attribute in the biochemical pathway of the different ML treatment interactions within the ovine parasite system, mechanisms underlying resistance may be understood. For example, over-expression of P-glycoproteins which mirror the function of a membrane efflux pump is thought to be partially responsible for both the moxidectin and ivermectin resistance seen in *H. contortus*

strains (Molento *et al.,* 1999). Therefore, investigation into cross- resistance in *P. ovis* is paramount in understanding the mechanism of resistant strains.

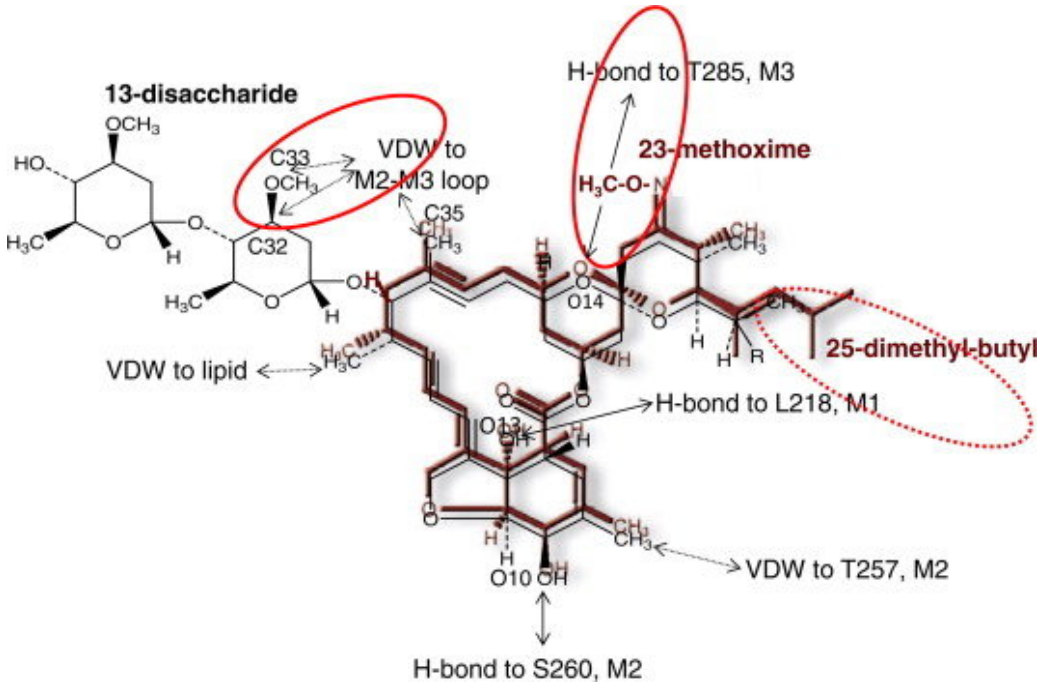


Fig. 1.5. Interaction of ivermectin (IVM) with a glutamate-gated chloride channel (GluCl) as proposed by Hibbs and Gouaux (2011), showing moxidectin (MOX; maroon) superimposed over IVM (black). Note that while some of the interaction sites are shared in common between MOX and IVM (O10, O13, C18, C35 and C48), other interaction sites of IVM with the GluCl are absent (C32, C33; both forming van der Waal (VDW) interactions with the GluCl in the case of IVM) due to the absence of any saccharide group in MOX, are blocked/altered (O14; H-bond in the case of IVM) by the C23 methoxime group of MOX.

The sites where interactions will be different for MOX compared with IVM are highlighted by a solid red circle. The 25-dimethly-butyl of MOX may also affect interaction with the GluCl (dashed red circle). IVM is a mixture of C25 ethyl (B1a; ~10%) and C25 methyl (B1b; ~90%). Against nematodes the B1b component is usually more potent than the B1a component, showing that the change from methyl to ethyl at this position affects potency. Thus, it is likely that the 25-dimethyl-butyl group of MOX will also affect the interaction with a GluCl. from Prichard, R. *et al.* (2012).

1.3.3 Future control development

Future use of the current acaricidal compounds is highly uncertain, given the effects of exposure on treatment operators and the environment described, coupled with the evidence of parasite resistance. Continued cooperation and education between veterinary surgeries and farmers to employ strict biosecurity in flock handling and movement is the first stage in scab control. However, management by biosecurity alone is unlikely to be practical. Therefore, the development of novel biological and chemical control methods has received much attention from the research community over the past two decades in the aim of finding a working and sustainable control method. Proposed alternative biological treatments include selective breeding for genetic resistance in sheep breeds showing low susceptibility, entomopathogenic fungi (Abolins *et al*., 2007), essential oils (Perrucci *et al.,* 1995) as well as genetically modifying microorganisms that exhibit commensal relationships with *Psoroptes,* such as *Bacillus thuringiensis,* to produce toxins that are fatal for the parasite (Smith *et al.,* 2001). However, to date none of these approaches has been sufficiently effective to lead to commercial development. Finding more effective, higher welfare and economical control methods for scab is important for future long-term management (Dunn *et al.,* 2016).

Development of an effective vaccination would provide many economic and safety benefits. Presently, attempts at creating a vaccine have only provided partial protection, reducing mite abundance and slowing lesion growth by >65% in re-infections. Three vaccine antibody trials were tested by Smith and Pettit (2004) using soluble *P. ovis* extracts, made through the homogenisation of frozen mite material in a phosphate saline buffer. During trials they found that even natural innate resistance to infection in sheep was only temporarily protective. The practicality of developing a vaccine is also extremely difficult and expensive as *P. ovis* cannot be cultured *in vitro* (Burgess *et al.*, 2016).

1.4 Aims and Objectives

Loss of drug effectiveness on parasite organisms is considered control failure. Studying field resistance can act as an important tool to establish biochemical action sites of resistance mechanisms. Field resistance studies on an organism are an essential complement to laboratory molecular studies. In part, this is due to factors in laboratory studies that are not reflected in the field and the need for both to discover, understand and target biological sites that confer resistance within parasite physiology (Sangster *et al.,* 2006). For example, resistance drift is seen within laboratory culture for ivermectin sensitive *H. contortus* which, as discussed (See Chapter 1.3.2), has been an important research organism for understanding the genomics of ivermectin resistance in ovine parasitic helminths (Gill & Lacey, 1998).

In light of the recent reports of *P. ovis* outbreaks not responding to ML treatment and the confirmation of moxidectin resistant *Psoroptes* organisms in the UK; this study aimed to determine whether outbreak populations of *P. ovis* that appeared unresponsive to treatment with moxidectin-based compounds, were also resistant or tolerant to the other compounds in this class. The response of seemingly tolerant *P. ovis* outbreaks to the milbemycin, moxidectin was explored, alongside responses to the macrocyclic lactones ivermectin and doramectin. The findings will identify if there is resistance to all three licensed ML-based compounds used to treat *P. ovis* in the UK. Therefore, indicating any necessary areas of research emphasis needed for the intervention and development of new control initiatives against the possible threat of cross-resistance generations of *P. ovis*.

CHAPTER 2

SHEEP SCAB RESISTANCE IN GREAT BRITAIN

2.1 INTRODUCTION

The fact that resistance has been reported in *Psoroptes ovis* to the milbemycin, moxidectin (Doherty *et al.,* 2018), while of concern, does not necessarily mean that resistance to the other macrocyclic lactones is inevitable. Macrocyclic lactones have different pharmacological profiles in different mammalian hosts when infected with *P. ovis*. For example, the oral bioavailability of moxidectin is almost 3 times lower in goat species and it has a 2.3 times shorter half-life in plasma than when administered to sheep breeds (Steel, 1993; Baynes *et al*., 2000). Moxidectin is also almost 100 times more lipophillic than ivermectin. The aqueous compound formula of moxidectin is more readily absorbed into the bloodstream than its oil-based ML counterparts. Seven days after subcutaneous injection, moxidectin has a 16:7 fat to liver storage ratio opposed to that of ivermectin which is 1:7 (Hayes, 1994). Moxidectin is known to transfer from fat into the liver for up to 28 days post-oral treatment. Sheep show a more gradual increase in moxidectin blood concentration over time than cattle which is suspected to be due to a slower turnover of lipids seen in sheep (Afzal *et al*., 1994; Zulalian *et al*., 1994). Hence, these known pharmacological differences may result in difference in their effect on mite populations and so cross-resistance cannot be simply assumed.

2.2. MATERIALS AND METHODS

2.2.1- Origin of mites

In this study, samples of naïve control populations of *P. ovis* mites were obtained from the Moredun Research Institute (MRI), Edinburgh. Here *P. ovis* mites were maintained on sheep that had had no previous exposure to acaricidal treatment. The mites have been in continuous culture without exposure to acaricide for at

least 10 years. Four samples were obtained from the MRI, Edinburgh for experimental work on 15/11/2017, 20/12/2017, 02/02/2018 and 17/03/2018.

Samples of field outbreaks that did not appear to have responded to standard acaricidal treatment were collected from veterinary surgeries from across the UK or posted by the veterinarians. Veterinarians or farmers with non-responding flocks contacted the study investigator through a network of veterinary contacts, and through contacts arranged by Zoetis Animal Health or Public Health England (Sian Mitchell). Collection was pre-arranged and posted samples were sent by special delivery, ensuring mites were off-host for less than 24 h between the skin scrape/collection and being used in bioassays. In total, 12 field samples were collected for the trials, but only 6 contained sufficiently large numbers of mites to allow their use**.** Mite sample origin and treatment history are detailed in Appendix B and Appendix C.

2.2.2 Bioassay development and Sampling (naïve and resistant)

The bioassay used to test for resistance was developed from the methodology of Doherty *et al.* (2018). Sterile petri-dishes (90 mm diameter) were partially filled with 18ml of potato dextrose agar (agar 15g/L; dextrose 20g/L, potato extract 4g/L, Sigma-Aldrich Ltd). On solidification, a surface layer of 1ml 5% Sigma-Aldrich sterile-filtered sheep serum and 1ml treatment solution was added. After adding mites to the centre of the petri-dish, the inner rim of the petri-dishes and lids were smeared with petroleum jelly and sealed with parafilm to ensure that the mites were contained.

In the current study, initial bioassay trials using MRI samples were used to confirm lethal conditions in which to test *P. ovis* mortality rate. The initial concentrations tested were 500ng/ml, 1000 ng/ml and 2000 ng/ml respectively, using the aforementioned study by Doherty *et al.* (2018) as a guide for concentration effectiveness. Under the experimental conditions of the present study bioassay, 2000ng/ml was found to be effective, resulting in 100% mortality in MRI naïve population samples under moxidectin treatment. Under the other MLs being assessed in this study for cross-resistance testing, the initial MRI samples did not

reach 100% mortality at the 72h time point used for analysis (See Chapter 2.2.3). This result of the 2000ng/ml precursor assays is reflected in the results of the MRI mortality rate during trial bioassays (Fig. 2.4). An additional concentration was added, and the experimental trials were run using 2000ng/ml and 4000ng/ml. Three MLs were used in the bioassays (Table 2.1), diluted in 100% ethanol to achieve the concentrations and lower concentrations used were abandoned for use in bioassay analysis due to the sample size required to test more than 2 concentrations adequately.

Negative control plates were made in an identical manner for each trial with 1ml absolute ethanol in place of the ML. Each concentration and ML treatment had 3 identical repeat plates with 10 mites placed on each plate for each trial. Before being added to a plate, the mites were tested for a locomotory response using tactile stimulation. Only mites capable of a full appendage contraction within 5 seconds of stimulation using 3 untreated paintbrush strokes across legs I and II (L and R lateral side) and the gnathosoma were added to plates. This ensured all mites used were alive and capable of movement before treatment exposure.

Table 2.1. The macrocyclic lactone, trade name, therapeutic dose and manufacturer of the compounds used in bioassays are reported here.

Macrocyclic lactone	Treatment ML Source
ivermectin	Oramec Sheep Drench, 0.08% w/v (Oramec)
moxidectin	Cydectin 20mg/ml Long Acting (Zoetis Animal Health)
doramectin	Dectomax 10mg/ml (Elanco Animal Health)

Field and control samples of infected skin and wool were inspected using a stereomicroscope (Leica S6E) and mites were transferred to plates using paintbrushes. Each treatment and concentration had specific brushes used for mite

transfer to prevent the contamination of individual organisms with other MLs or concentrations. After all mites were placed on the bioassay plates for a trial, the plates were sealed and incubated at 20^{o}C and 80% r.h. Smith *et al.* (1999) showed that *Psoroptes* survival was reduced at relative humidities below 75% r.h. and a study by Brimer *et al.*(1995) demonstrated that MLs remain stable and active in agar plates at 20 °C over the duration of bioassay trials.

Appendix D details a letter sent by the study investigator through the study contact network, after receiving samples that were not appropriate for bioassay analysis. Pre-assay mite mortality occurred due to desiccation in many samples until sampling instructions had been communicated with Veterinary and Animal health suppliers. The letter details the optimum sampling method and outbreak sample format based on initial pre-assay trials. For a successful trial, bioassay materials needed to be set up before an outbreak sample arrived at the laboratory.

Within the desiccation time-sensitive window of < 4 hours, the treatment solution, sheep serum and *P. ovis* mites were placed on each assay plate. Plates were then incubated at the optimal relative humidity and temperature. In large samples, with many containers, only a single container was used at one time and the remainder of the sample were kept incubated to prevent mite desiccation.

Mortality counts were recorded for all bioassay plates at 24 h intervals for 120 h after initial setup. A mite was categorised as alive based on tactile stimulation and locomotory response, as described previously. Again, treatment specific treatment brushes were used to avoid cross-contamination of treatments/concentration and brushes were rinsed in 100% ethanol in-between tests.

2.2.3 Statistics

The outbreak trial and control trial results were entered into Excel. The plate number, drug, concentration and number of live *P. ovis* mites at each 24h interval were recorded. The percentage mortality for each plate and the median percentage mortality for each treatment were calculated in both the outbreak and the control populations. Non-parametric statistics were used throughout as the data were not normally distributed, as confirmed using a Shapiro-Wilk W test (P

Value< 0.05). R statistical software and Statgraphics were used to analyse the data. The final significant differences in % mortality for the 2000ng/ml and 4000ng/ml concentration trials were calculated using StatGraphics Centurion 18 software (Statgraphics, 2018; R Core Team, 2013) by performing Mann-Whitney U tests. A P value of <0.05 was considered significant.

Mortality comparison statistics were taken at 72h for comparison between the field and naïve MRI control samples. The 72h time point was used due to control tests showing that under the optimal humidity and temperature conditions used in this study, there were no significant difference in % mite mortality between field (outbreak) and naïve control (MRI) population samples at 72h when exposed to ethanol and sheep serum (nutrition) only (W=26.0, P Value> 0.05). Initial pre-assay testing demonstrated that using this time point ensured that both control and outbreak mite mortality rates were due to experimental treatment conditions rather than time subject to off-host conditions.

2.3 RESULTS

2.3.1- Response of populations to control treatment bioassay

Ethanol controls:

When exposed to ethanol only, mites from both outbreak and MRI samples showed low mortality which, as expected, gradually increased with time subject to off host conditions. At 72 h after initial exposure, there was no significant difference in the % mortality between the MRI or outbreak populations (W= 26.0, P Value> 0.05).

2.3.2- Response of Field and Control *P. ovis* mite samples to macrocyclic lactone bioassay

Appendix E details a summary table of statistical differences between % mortality responses at 72h in ML treated and untreated control samples for both outbreak and MRI sample groups.

Moxidectin:

At 72h post exposure to 4000 ng/ml of moxidectin, both the outbreak and the MRI samples had a median % mortality of 100% (Fig. 2.3). When contrasting the response of MRI bioassays (Fig. 2.1; Fig. 2.3) to that of outbreak bioassays (Fig. 2.2; Fig. 2.3), MRI sample responses to 4000ng/ml moxidectin exposure were all >80% mortality, whereas the response of seemingly tolerant outbreak samples varied across the range, with two samples <70% mortality at 72h. Study on the blood plasma concentration of moxidectin in treated sheep found that at 12h post-treatment, blood plasma concentrations peak at a maximum of 20ng/ml. By these findings (Lloberas *et al.,* 2013), after exposure to the 4000ng/ml bioassay in the current study, the mites were subject to a 200-fold increase in concentration relative to standard dosages of veterinary treatment in the field.

Using the Mann Whitney-U test for significance analysis, in the outbreak samples, there were significant differences seen in % mortality response to Moxidectin 4000ng/ml and the outbreak sample control mites exposed to ethanol and sheep serum nutrition only ($W=0$, P Value< 0.05).

When exposed to a 2000 ng/ml concentration of moxidectin, the median % mortality in the field outbreak samples was half that of the MRI mite populations (Fig. 2.4). Insufficient outbreak data were obtained after outbreak moxidectin exposure at this concentration for statistical analysis. However, the results obtained (Fig. 2.1; Fig.2.2), imply considerable disparity between the median % mortalities of outbreak and MRI sample responses.

In the MRI naïve mites, there were significant differences seen in % mortality response to Moxidectin 4000ng/ml and the control MRI mites exposed to ethanol and sheep serum nutrition only ($W=0$, P Value< 0.05). There were also significant differences in % mortality to MRI mites exposed to the lower 2000ng/ml concentration and MRI control mites exposed to ethanol and nutritional serum only ($W=0$, P Value< 0.05).

Doramectin:

After exposure to doramectin 4000 ng/ml treatment, the results also imply considerable disparity between the sample group responses (Fig. 2.1; Fig. 2.2). At 72h, the MRI samples median % mortality is 100%, as opposed to the outbreak samples responses, with a median % mortality of 15% (Fig. 2.3). Dissimilarity between responses of outbreak samples and the MRI samples is seen after exposure to 2000ng/ml doramectin also (Fig. 2.4). After 4000ng/ml exposure the difference in % median mortality between outbreak and MRI sample response was 85% lower in the outbreak samples compared with that of the MRI naïve samples. After exposure to 2000ng/ml, the difference was a 40% lower median mortality in outbreak samples than in the MRI samples.

In the outbreak samples, there were no significant differences seen between % mortality response to Doramectin 4000ng/ml and the response of outbreak sample mites exposed to ethanol and sheep serum nutrition only ($W=9.5$, P Value >0.05). There were insufficient outbreak data after exposure to 2000ng/ml Doramectin for statistical comparisons at this concentration between outbreak treated and control groups.

In the MRI naïve mites, there were significant differences seen in % mortality response to Doramectin 4000ng/ml and the control MRI mites exposed to ethanol and sheep serum nutrition only ($W= 0$, P Value <0.05). There were also significant differences in % mortality to MRI mites exposed to the lower 2000ng/ml concentration and MRI control mites exposed to ethanol and nutritional serum only ($W= 0$, P Value <0.05).

Obtaining a larger data set through larger outbreak *P. ovis* samples would allow for full statistical analysis of these differences. The study objectives would benefit from a larger sample size for increased statistical confidence and reduced margin of error when contrasting responses to MLs between non-responding samples and the naïve MRI samples. Larger samples would allow more data on multiple ML testing within a single outbreak sample and so an in-depth analysis of significant differences between ML responses could be performed. This could indicate differences in susceptibility levels to each ML within outbreak groups and provide

evidence towards investigating shared biochemical target sites between the MLs and parasite physiology which confer resistance as previously discussed (See Chapter 1.4).

Ivermectin:

After exposure to 4000 ng/ml ivermectin, there were significant differences in the % mortality seen at 72h between the outbreak and MRI naive samples (W=15.0, P Value <0.05; Fig. 2.1; 2.2; 2.3). Treated and untreated MRI naïve samples also had a significant difference in % mortality at 4000ng/ml (W=0, P value<0.05) (Fig. 2.1). There was no significant difference between the treated and untreated MRI samples after exposure to the lower 2000ng/ml concentration of ivermectin treatment (W= 0, P Value >0.05).

There were also no significant differences between the % mortality seen in the outbreak samples exposed to ivermectin at either concentration when compared with the outbreak sample control mites exposed to ethanol and sheep serum nutrition only (4000ng/ml: W=18.5, P Value >0.05; 2000ng/ml: W= 8.0, P Value >0.05; Fig. 2.2).

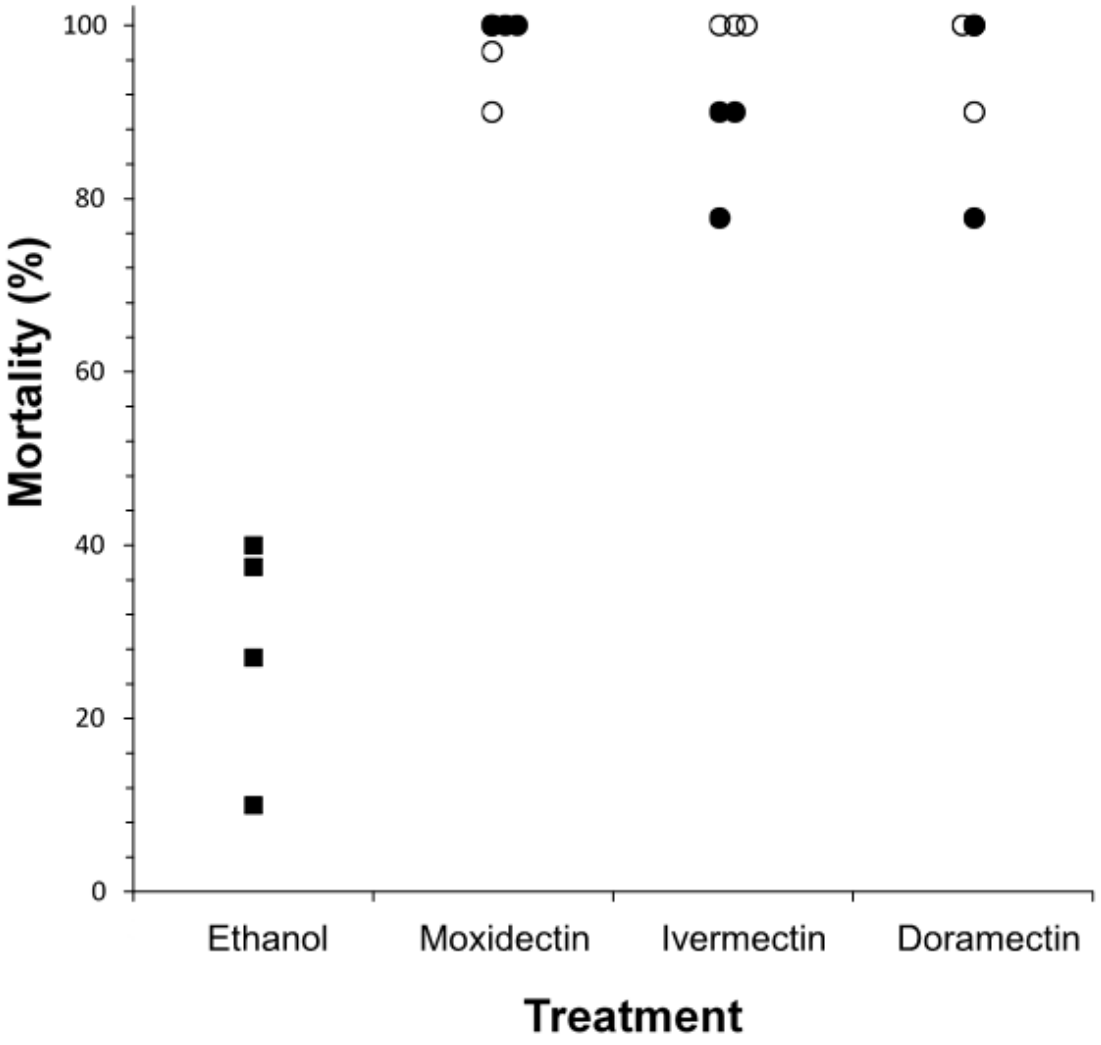


Fig. 2.1. The percentage mortality of *Psoroptes ovis* obtained from the Moredun Research Institute and exposed for 72 h to agar and sheep serum plates, treated with ethanol (squares) or moxidectin, ivermectin or doramectin at 2000 ng/ml (solid circles) or 4000ng/ml (open circles).

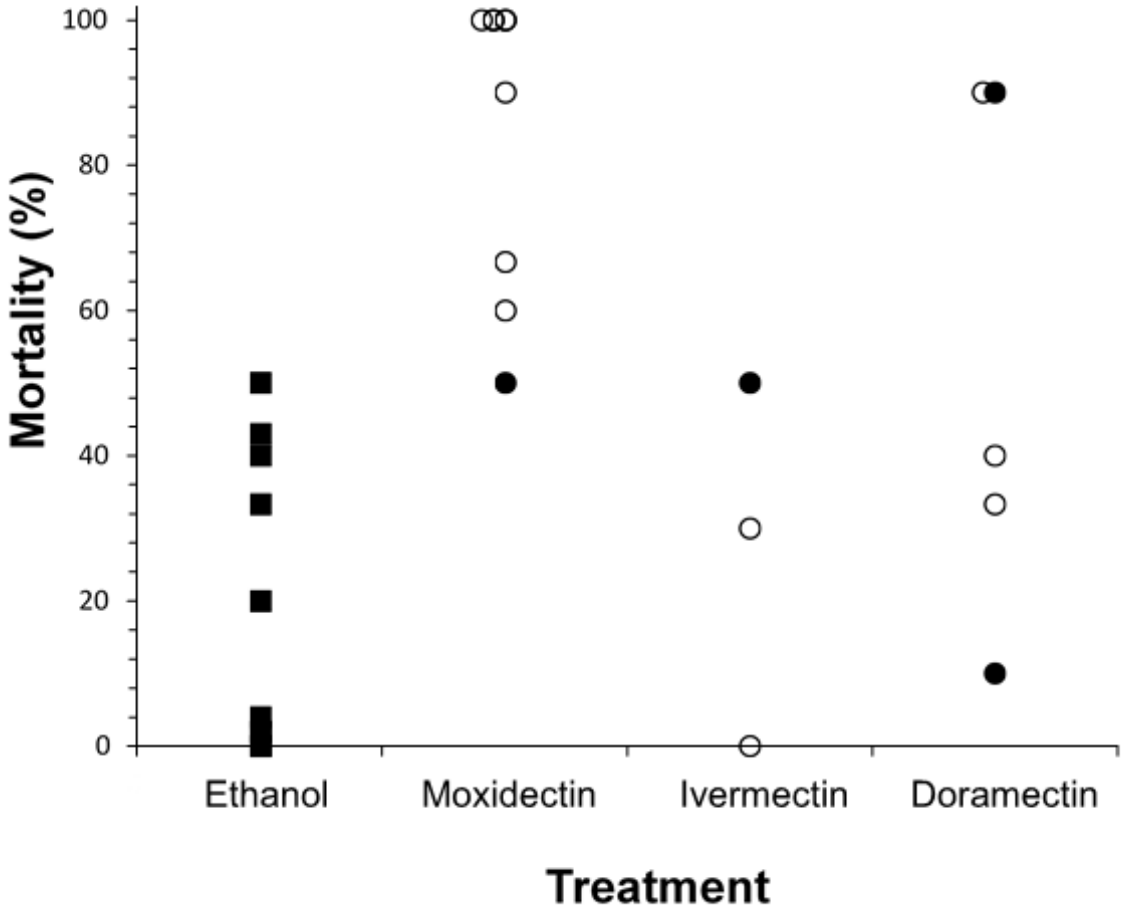


Fig. 2.2. The percentage mortality of *Psoroptes ovis* obtained from non-responding outbreak samples and exposed for 72 h to agar and sheep serum plates, treated with ethanol (squares) or moxidectin, ivermectin or doramectin at 2000 ng/ml (solid circles) or 4000ng/ml (open circles).

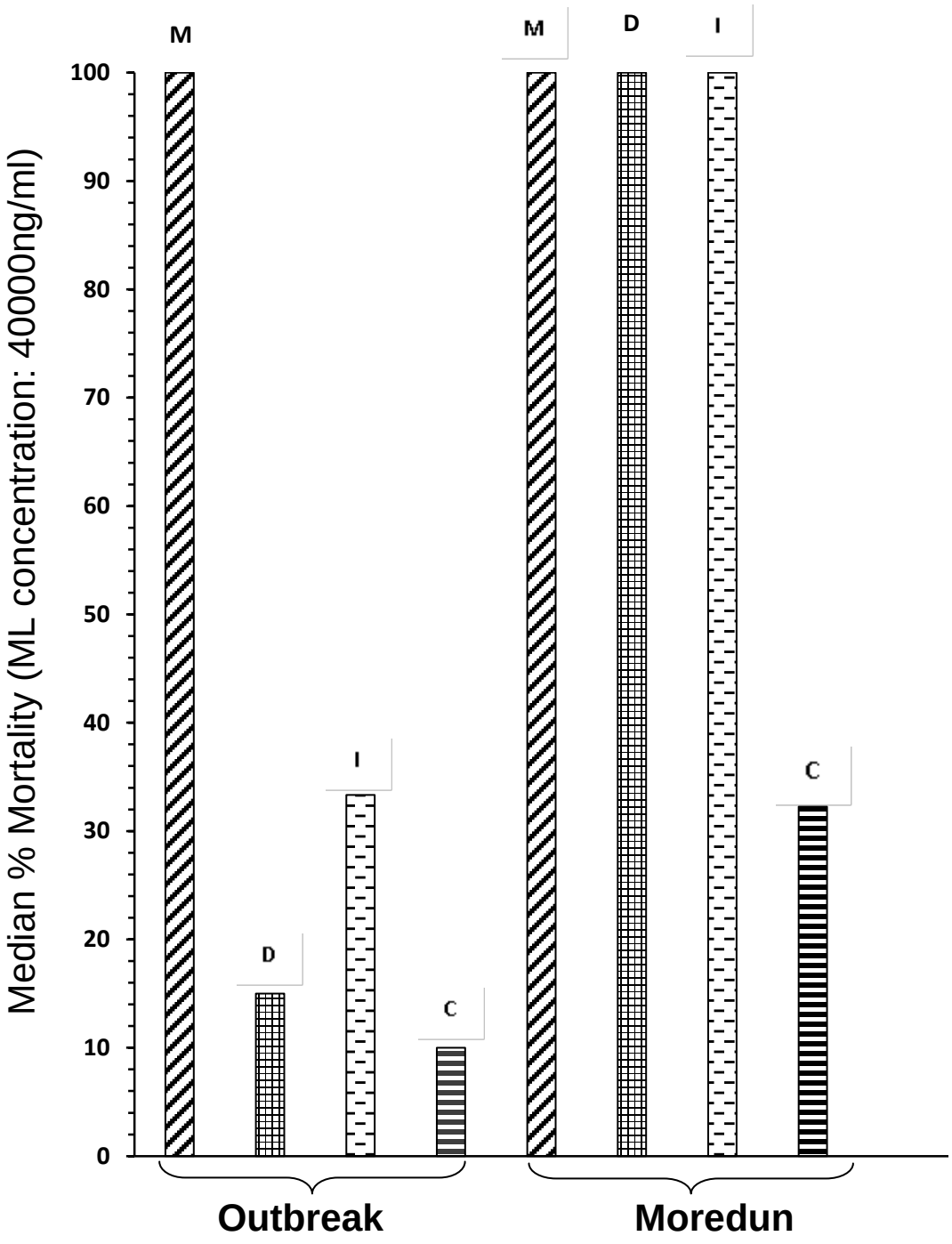


Fig. 2.3. **Median percentage mortality of *Psoroptes ovis* outbreak population samples and Moredun Research Institute naïve control population samples exposed to macrocyclic lactone treatments at 4000ng/ml. Macrocyclic lactones: moxidectin (M), doramectin (D), ivermectin (I) Untreated controls exposed to 100% ethanol and sheep serum only (C)** .

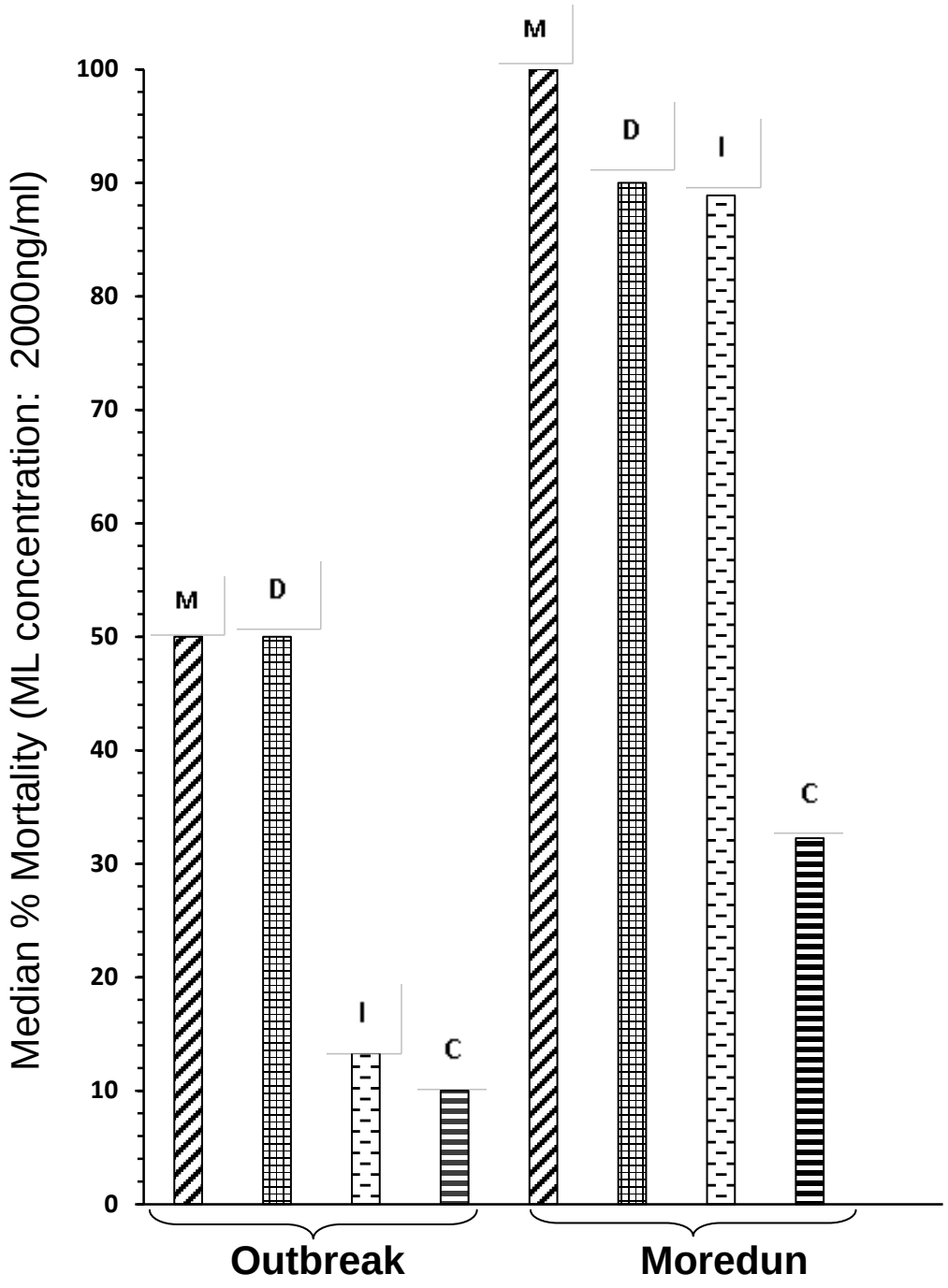


Fig. 2.4. Median percentage mortality of *Psoroptes ovis* outbreak population samples and Moredun Research Institute naïve control population samples exposed to macrocyclic lactone treatments at 2000ng/ml. Macrocyclic lactones: moxidectin (M), doramectin (D), ivermectin (I) Untreated controls exposed to 100% ethanol and sheep serum only (C).

Results summary:

There were no significant differences found between the tolerant outbreak samples and ML naïve MRI samples when exposed to moxidectin or doramectin at 4000ng/ml (Moxidectin: P Value >0.05) (Doramectin: P Value >0.05). This is arguably expected for moxidectin as it is known to be the most effective ML treatment against *P. ovis* and particularly effective in parasites showing resistance to avermectin treatments (Prichard *et al.,* 2012). The mites were exposed to the highest laboratory concentration which is much higher than the concentrations that in-field *P. ovis* infections would be exposed to, through standard treatment dosage (See Chapter 2.3.2 "*Moxidectin"*). It appears that the moxidectin 2000ng/ml data also commensurate the findings of the Doherty *et al.* (2018) study discussed previously (See Chapter 1.3.2), in that outbreak samples appear to have a much lower mortality % than those of MRI samples exposed to the same treatment (Fig. 2.4). A larger sample size would be necessary to explore the statistical validity of this phenomenon as with the doramectin exposure outbreak responses at the lower 2000ng/ml concentration (See Chapter 2.3.2 "*Doramectin"*). The higher concentration was preferred with the low sample sizes of the current study. As discussed, this was based on the limitations and impacts of the off-host environment on natural mortality and mite condition and sample sizes, seen under the pre-assay trials of the study methodology (See Chapter 2.2.2). 4000ng/ml was favoured in each individual bioassay in order to acquire sufficient data for analysis under the highest concentration.

Surprisingly, despite a significant difference seen between treated and untreated outbreak samples when exposed to 4000ng/ml moxidectin, there is no significant difference between treated and untreated outbreak control sample response after exposure to 4000ng/ml doramectin. Paired with the evidence of a reduced response in outbreak samples to both moxidectin and doramectin 4000ng/ml in comparison to the % median mortality in MRI sample responses (Fig. 2.3), this data indicates that non-responding outbreak populations show a high level of resistance to doramectin and maintain the natural mortality rate under treatment with doramectin-based ML compounds. The results of the % mortality after ivermectin

exposure insinuate that it was the least effective ML used in this study based on the % mortality response of both outbreak and MRI *P. ovis* samples. However, the MRI samples show a consistently high mortality to both ivermectin concentrations (Fig. 2.1). There was a significant difference between the MRI samples treated with 4000ng/ml and the untreated mites, but this is not the case at 2000ng/ml, suggesting that the threshold for Ivermectin to be effective on *P. ovis* responsive mortality requires a very high concentration. The significant difference seen between treated outbreak samples and MRI naïve samples after 4000ng/ml ivermectin exposure is reflected visually in the figures (Fig. 2.1; Fig. 2.2; Fig. 2.3; Fig. 2.4) and represented in Appendix E.

2.4 DISCUSSION

The results of this study must be interpreted with care. Outbreak populations come from farms that reported that their infestation of scab had not responded to treatment. But lack of response may be due to a variety of factors, one of which may be resistance, but another may simply be improper application of the acaricide used. Hence, it should not be surprising that some of the outbreak populations did appear to be susceptible to the ML against which they were tested. For example, in the trials with moxidectin at a concentration of 4000 ng/ml most of the mites tested appeared to be susceptible, suggesting that no resistance was present. However, upon closer inspection it can be seen that 3 of the outbreak samples exhibited lower % mortality than the MRI control samples (Fig.2.1; Fig. 2.2), while the others did not. Differing responses may also be attributed to the fact that each non-responding outbreak sample had a different previous treatment history. Furthermore, the study was only able to obtain mites from a relatively small number of outbreak populations, and in most of these cases only small numbers of mites could be obtained from the wool and scab samples submitted.

The concentrations of the MLs used in the bioassays are significantly higher than the plasma concentrations that a mite would be expected to encounter in serum on a sheep and higher that those used by Doherty *et al.* (2018). These high

concentrations were selected to maximise the challenge presented to each exposed mite and give confidence to the conclusion that mites were or were not resistant to the compound. Whilst the mechanism of resistance are not currently known, the MRI naïve samples % mortalities after treatment compared with that of the less susceptible outbreak samples, demonstrated a clear difference in response. The phenomena that some outbreak samples proved less susceptible to the lower 2000ng/ml moxidectin and doramectin concentrations than the MRI samples (Fig. 2.4), whilst still showing a high % median mortality to the 4000ng/ml concentrations (Fig. 2.3) may indicate that there is some biochemical level of mortality threshold and partial resistance in unresponsive cases of sheep scab. Some cases of ML resistance in other parasitic species are conferred through a lower binding affinity to ML treatments at glutamate-gated chloride channel subunits (McCavera *et al.,* 2007). In the case of a resistance mechanism that is related to a lowered binding affinity, it is possible that a higher concentration would cause enough instances of treatment chemical interaction with the action site, that the chances of an active chemical bond forming are higher.

Another example is that of resistance to moxidectin and ivermectin conferred through genes coding P-glycoprotein expression seen in strains of *Caenorhabditis elegans* (Bygarski *et al.,* 2014), which as previously discussed (See Chapter 1.3.2) also confers resistance in *H. contortus.* Therefore, the results in this study may also indicate that the higher treatment concentrations can overcome this tolerance in *P. ovis* if resistance is conferred by the same biochemical target mechanisms. However, the restrictions of safe concentration levels in veterinary treatment would impede solutions to this that use concentration levels alone. An increased understanding in the biochemical target sites of resistance could guide the development of novel treatments with a different mechanism of action and biochemical interaction.

The general pattern of mortality seen in this study strongly implies there is resistance to all 3 common ML treatments in many of the non-responding outbreak populations, when their mortality is compared with the much higher levels of mortality seen in the naïve control samples obtained from the MRI populations.

Where outbreak samples did show susceptibility to the test MLs it suggests that environmental factors, such as ineffective or inappropriate application technique or repeated infection, particularly in the case of ivermectin where there is no residual activity, are likely to be responsible for the persistent scab. *P. ovis* have been reported as surviving for up to 38 days off host, although survival is strongly seasonal (Babcock and Black, 1933). Mites have been shown to retain infectivity off host for at least 15 days. Adult females survive longer than males and all mites survive longer off-host at cooler temperatures. Eggs are also viable for a week in an off-host environment (Shilston, 1915; O'Brien *et al.,* 1994). Therefore, all equipment, land and territory instalments e.g. fencing/walls that enclose an area with infected individuals, must be considered as a re-infection source and the flock must be quarantined. There are also accounts of under-dosing or weight measurement errors, resulting in ineffective doses of ML being administered as treatment (O'Brien, 1999). Above are some examples of the more common reasons that could result in a case of false suspected resistance.

During the study, the most common ML for which resistance was suspected was moxidectin. In the previous study assessing resistance in *P. ovis,* only moxidectin was considered and outbreak populations showed 20%-30% mortality at 72h after exposure to a 2000ng/ml concentration of moxidectin treatment (Doherty *et al.,* 2018). In this study, the median mortality in outbreak populations was 50% at 72 h after exposure to the same concentration of moxidectin. Given the sample sizes the difference in mortality between the studies is not unexpected, but additional slight differences between the methodologies used in the studies might have also contributed to the difference. Mites were exposed to treatment directly through a surface layer of treatment plate solution in the current study, rather than indirect uptake through the treatment mix within plate agar. The method of treatment uptake has not been conclusively defined. However, whether ingested through the gnathosoma (feeding organs) of the mites or absorbed through the cuticle surface directly, direct treatment exposure would expectedly result in a higher absorption, thus higher saturation or ingestion of the treatment chemical.

There are many other factors that may affect treatment efficacy in addition to resistance that should be considered. It was thought that contributing factors which impact sheep-scab treatment response in the field may include the age and sex of the infected sheep, however, upon investigation, there were no statistically significant differences between infection prevalence and age or sex of infected individuals (Tasawar *et al.,* 2007). Although these compounds are highly stable and have high lipophilicity (Campbell and Benz, 1984; Prichard *et al.,* 2012; Vercruysse *et al.,* 2018) subtle variations in their formulation may affect their activity. It has been suggested that treatment efficacies of the same ML may vary between different products; it has been argued for example that generic products may be less effective than branded products and that the method of administration may impact the pharmokinetics and efficacy of some ML treatments (Eraslan *et al.,* 2010; Toscan *et al.,* 2012).

Nevertheless, in a trial, 22 cattle infected with *P. bovis* were allocated at random into groups. Two injectable brands of ivermectin were administered at an appropriate dosage for individual cattle weight. The cattle were subject to the same environmental conditions and the groups were kept separate to avoid cross-contamination or parasite transmission (Genchi *et al.,* 2008). No difference was found in efficacy or pharmacokinetic profile between two separate brands of ivermectin (Genchi *et al.,* 2008; Hamdullah *et al.,* 2015). Further study with more samples and experimental focus on treatment history would be required to quantify the effect of these factors on *P. ovis* mortality, including specific treatment brand and administration method.

The Insecticide Resistance Action Committee (*syn* IRAC) classifies acaricide modes of action into categories based on the physiological functions they target. These include respiratory, nervous system, growth and midgut targets. Avermectins and milbemycins are classified into the glutamate-gated chloride channel allosteric modulators. Twenty-five groups of known acaricide targets have been defined, with an additional group of 4 drugs of which the mode of action is unknown (IRAC, 2016). The Doherty *et al.* (2018) study was the first to demonstrate resistance of *P. ovis* to moxidectin, and so the physiochemical mechanisms of resistance have not

yet been identified. Nevertheless, the similar mode of action of macrocyclic lactones would be expected to confer cross-resistance across the class (Wolstenholme, 2012). The response of outbreak samples to doramectin and ivermectin trials in the current study provides further evidence of growing resistance in UK field populations across the macrocyclic lactone class of compounds. Percentage mortality responses of outbreak samples exposed to 4000ng/ml ivermectin and those exposed to 4000ng/ml doramectin were not significantly different from controls from the same outbreak samples exposed to ethanol-only which, as discussed (See Chapter 2.3.2 "*Results summary"*), indicates that non-responding outbreak populations demonstrate a high level of resistance to both compounds and maintained a natural mortality rate that mirrored the untreated mites after exposure to treatment.

CHAPTER 3

GENERAL DISCUSSION

Introduction:

Livestock ectoparasites are ubiquitous and it is usually extremely difficult or impossible to fully eradicate non-isolated populations (Wall, 2007). Management at a local level, and consistent integrated approaches, designed to maintain low regional prevalence, are usually necessary to minimise the economic and welfare consequences from these parasite species. In the case of *P. ovis* mites, the co-evolution and symbiotic association between the mites and their wild hosts, seen largely as relatively benign ear forms, has been disrupted in the last few thousand years, by the artificial selection of more highly productive sheep breeds in domestic farming and the introduction of an array of transmission factors associated with intensive animal husbandry (Stevens *et al.,* 2006). For example, a study by Rose *et al*. (2011), analysed sheep movement and ranging on common land finding that in Wales, 99% of a flock's range was shared with 1 or more other farms. This study identified common grazing as a significant transmission and risk factor for reoccurring scab outbreaks.

The evidence presented in this study strongly suggests that resistance to multiple MLs is present in UK based outbreaks of *P. ovis*, posing increased financial and welfare threats for the veterinary and agricultural community. At present, this appears to be largely confined to Wales and the Welsh-borders. If resistance is able to spread, scab prevalence in Britain would be expected to increase over the next decades (Doherty *et al.,* 2018).

Insecticide resistance:

The development of resistance to insecticides is influenced by a combination of factors, from genetic mechanisms underlying treatment interaction, through to the

frequency, dose and method of acaricide application. Studies have suggested that most resistant alleles are recessive and disadvantageous in the absence of endectocide; *in vitro* experiments have shown reduced reproductive ability, thus lower reproductive output in individuals with resistant alleles (Georghiou and Taylor, 1977). To more efficiently manage and prevent accumulation of resistance gene selection, the IRAC advocate seasonal rotations of acaricides/insecticides with different modes of action and target areas of parasite physiology (IRAC, 2016). In practice however, in the case of *P. ovis,* the lack of alternatives to MLs makes this difficult. Study into minimising selection for resistance to ivermectin, moxidectin and another ML, abamectin (an insecticide used for ovine helminth infections), found that even when rotating ML treatments and combining different drugs for treatment, it is unlikely to delay selection for ML resistance. The results of the study by Dobson *et al.* (2001) highlighted the importance of monitoring ML and drug combination efficacy through post-treatment parasite screening. The authors also concluded the importance of reducing ML reliance through avoiding regular annual use and treating conditions with lower efficacy drugs where appropriate.

Mechanisms of resistance and cross resistance:

Understanding mechanisms of ML resistance in *Psoroptes* mites is an important next research step, as it may be able to contribute to the development of novel working treatments, or biochemical modifications to current products. As discussed, the strongylid ovine parasite, *H. contortus,* has also shown resistance to MLs, which has been associated with the over-expression of P-glycoproteins (Molento *et al.,* 1999). Understanding resistance on a molecular level is essential to allow the identification of the site of action between the resistant parasite and the treatment chemical structure. This understanding can then guide the development of working treatment solutions. Molecular research into the changes in genome which confer resistance allow for biomarker recognition and the creation of early, asymptomatic diagnostic and treatment methods. Molecular research of resistant *H. contortus* strains, found polymorphism within the alleles, *glc-5* and *lgc-37*. *Glc-5* is a glutamate-gated Cl^- channel subunit and *Igc-3* is a subunit of the GABA-gated

chloride channel, both of which are targeted by MLs. The specific nucleotide change responsible is yet to be discovered, however, the alleles associated with ML resistance *in vitro*, were seen *in vivo* also to be related to adult feeding and larval locomotion (Beech *et al.,* 2010). An increase in resistant allele frequency in *H. contortus* populations, under treatment exposure, requires a selective pressure that favours a genetic advantage in the surviving members (Blackhall *et al.,* 1998). The information known for ML resistance mechanisms in other parasitic organisms may indicate target sites that would require molecular analysis and investigation in *P. ovis* mites which appear unsusceptible to treatment.

Perhaps molecular evidence on drug resistance in other arthropod Acari would provide an initial framework to guide research, given the closer taxonomic relatedness to *P. ovis* than that of *H. contortus*. For example, the important crop pest – the two-spotted spider mite, *Tetranychus urticae*, exhibits insensitivity to acaricide treatments. As with *P. ovis* the rapid life-cycle and high rate of reproduction of *T. urticae* facilitates the rapid development of resistance under acaricidal selection pressure (Van Leeuwen *et al.,* 2010). It is likely that *P. ovis* will show varying levels of resistance between populations and host variants. A study of 15 genetic variants of *T. urticae*, found differences in the levels of sensitivity to 8 acaricides in toxicity assays. Resistant strains were classified by a <50% mortality after exposure or recorded as highly resistant if mortality remained low at 500% of the discriminating dose. Two *T. urticae* variants demonstrating high resistance levels to acaricides were also found to resist cyflumetofen, a chemical that had not been used in the relevant regions of Europe before the study and so was not a compound the mites had been knowingly exposed to previously. This further implies the importance of understanding shared target mechanisms of resistance in parasite-treatment interaction. Other variants showed a wider spread of resistance to more common treatments (Khajehali *et al.,* 2011).

The demonstration of cross-resistance to compounds related to or chemically similar to the treatments organisms are insensitive to, has also been observed in *Drosophila* through a mutation resulting in gene upregulation. Insertions of transposable elements in the leader RNA sequence, 5'UTR regions, are well-

documented and transposition in the Cyp61 gene of *Drosophila* is particularly noteworthy in regard to cross-resistance. The enzyme metabolises neonicotinoids and DDT, thus a mutation resulting in upregulation of the encoding gene elicits cross-resistance to both compound classes (Daborn *et al.,* 2002; Schmidt *et al.,* 2010). In the Southern cattle tick, *Rhipicephalus microplus*, an economically important agricultural ectoparasite, resistance to MLs has been reported to result from an insensitivity of GABA or chloride channels. Altered acaricide sensitivity at biological target sites by genetic point mutations, cause a significant proportion of the cases of resistance development. Resistance to the ML, abamectin, has been shown to be usually autosomal, recessive, and determined by multiple gene inheritance. Resistance is conferred through a decrease in binding sites, especially at the GABA alpha subunits (cys-loop ligand-gated ion channels) of the GABA A receptor (Clark *et al.,* 1991; Clark *et al.,* 1995; Barnard *et al.,* 1998).

There are a group of known mutations in Acetylcholinesterase genes (AchE or 'Ace' genes) which provide resistance mechanisms to organophosphates and other insecticides in other species of ectoparasitic arthropods, including some species of mite and ticks. The molecular characterisation of these can be complicated because the number of AchE genes varies between arthropod species (Temeyer *et al*., 2004; Grbić *et al.,* 2011; Feyereisen *et al*., 2015). The ESTHER database has more than 12 records of sites in different organisms and a collection of polymorphisms that have mutant AchE genes, causing tolerance to insecticides (Lenfant *et al.,* 2013). Mutations in the GABA receptor gene coding region have been discovered in 27 arthropod species (Feyereisen *et al.,* 2015). The GABA-gated chloride channel subunit targeted by the insecticides, cyclodiene and phenylpyrazole, is encoded by the Rdl gene. A single-point mutation in this gene coding region, resulting in an amino acid replacement of Ala^{301} to Ser, confers cyclodiene resistance (Remnant *et al.,* 2013). Widespread use of cyclodiene during the 1980s, evoked insensitivity to the treatment in 62% of species that had demonstrated insecticide resistance (Georghiou, 1986).

As previously mentioned (See Chapter 1.3.3), developments into a vaccine have only proven partially protective against *P. ovis.* The MRI division working on a

vaccine have published the first draft genome of the *P. ovis* mite (Burgess *et al.,* 2018). This development in molecular understanding may act as a valuable resource for genetic understanding and as a comparison genome between samples which respond and samples which appear unresponsive to treatment, to find any point mutations in *P. ovis* which confer ML resistance. Presently, there has been further development in molecular understanding of acaricide insensitivity in *T. urticae* than in *P. ovis,* and as mentioned, understanding resistance target sites in closely related organisms is likely a fundamental step in finding novel control methods for resistant *P. ovis.*

Homologues of RNAi transcript sequences in the *T. urticae* genome have recently been identified and these have led to successful gene silencing attempts and a substantial reduction in transcript levels of three target sequences which encode the sheep scab allergen protein, Pso o 2, in *P. ovis* (Marr *et al.,* 2018). This further emphasises the importance of comprehensive molecular analysis of resistance mechanisms and shared chemical characteristics between treatment compounds. As well as using shared biochemical features between the physiological interactions with compounds in different species that appear insensitive to same insecticides, as a reference for investigation into resistance mechanisms.

Current state of Psoroptes ovis management in the United Kingdom:

It can be argued that the current UK approach to *P. ovis* control is unreliable and unsustainable. Persistent outbreaks arise from inappropriate or ineffective treatment, inconsistency of treatment between in-contact farms and/or poor biosecurity leading to repeated reinfection (Rose *et al*., 2011). Furthermore, the absence of nationwide surveillance means that inadequate data are available to track scab incidence, since data collection within academic research projects results in infrequent and out-of-date prevalence estimates. Following reintroduction of the disease, the lack of coherent national management allowed scab to spread and become more prevalent. The increase in prevalence was also helped by repeated infection particularly from feral sheep on common land acting as reservoirs (Roberts and Meleney, 1971), the lower residual activity of organophosphates

compared to organochlorines, in addition to the fact that some mites were able to escape dip through inhabiting "cryptic (or latent) sites". For example, *P. ovis* infections in ear canals where dip has been shown to be unable to penetrate (Kirkwood and Quick, 1978). These factors all contributed to the inability to re-eradicate scab (Bates, 1991; Sargison and Busin, 2014). In addition, considerable misunderstanding within the sheep farming community on the appropriate types and timing of treatment has been demonstrated (Bisdorff and Wall, 2007). The latter survey showed that prophylaxis treatment for scab was often given during summer months, which is ineffective for protection because the peak outbreak period is in winter and inappropriate insecticide were used, such as the insect growth regulator cyromazine (Bisdorff and Wall, 2007). Now, with the development of resistance to macrocyclic lactones, the management of scab is likely to be considerably more difficult and the range of potentially useful acaricides is now much more limited. Paradoxically, the presence of resistance in *Psoroptes* to MLs may help slow the development of resistance to these compounds in GI nematodes, since it should eventually reduce their use against scab, and the unnecessary concomitant exposure of worms. As previously discussed (See Chapter 1.3.1), treatment for ovine worm infections and other ovine diseases can inadvertently expose undetected sub-clinical scab to low-level ML doses, provoking resistant gene selection (Coles, 1998).

Five of the six outbreak samples of the working trials of this project, contained a large enough number of *P. ovis* mites to allow tests against more than one ML in a single bioassay trial. Of these five, three demonstrated resistance to more than one ML. A thorough, replicated assay with much larger sample sizes from non-responding *P. ovis* outbreaks would be necessary to demonstrate cross-resistance conclusively. Obtaining such large numbers of mites from clinical cases, however, is difficult.

Future directions to manage resistant scab:

To date, as seen in this study, most reports of resistant outbreaks are located in Wales and the Welsh borders (Doherty *et al.,* 2018). In terms of the future

management of scab in the UK, analysis by French and Wall (1999) indicated clear associations between changes in government scab management policy and scab prevalence, demonstrating the impact and importance of central government-led policy in national management. The analysis also identified local space-time clustering, with higher levels of incidence within a five-month time span in farms in close proximity to each other, demonstrating the importance of contact between infected flocks in scab transmission. This is supported by data showing that scab risk is highest on farms that use regional common grazing (Rose *et al*., 2011; Chivers *et al.,* 2018). This is an important consideration for policy formation looking at minimising the spread of resistant outbreaks of *P. ovis.* Other considerations that need to be included in the context of scab management include the behaviour of farmers and the economic justification and incentives supporting prophylactic treatment. A game-theory model analysing current national scab trends showed that prophylactic treatment is only economically favourable in upland farms where scab prevalence is highest. The model shows that prophylactic treatment would need government subsidy in lower-risk areas for high levels of compliance through being cost effective for farmers (Nixon *et al.,* 2018). In the future, it may be possible to slow the spread of resistant genes across the country, although this will be difficult. Genetic biomarkers for resistance would be useful to help identify resistant populations quickly and effectively, particularly at the asymptomatic stages of infection.

Recently, ELISA tests (enzyme-linked immunosorbent assays) have been developed and marketed, that are able to detect sub-clinical scab for rapid diagnosis. The first assay was developed in 2001, modified from a previous assay used for *P. bovis* detection (as described by Lonneux *et al.,* 1996), but it has been continuously improved since this time. A subsequent version of the ELISA had a 96.5% specificity and 93.7% sensitivity in clinically presenting sheep with moderate levels of detection 2 weeks before clinical signs (Ochs *et al.,* 2001). This assay has been further refined to give a sensitivity of 98.2% and specificity of 96.5% (*pers comm.* S.Burgess, MRI). Application and further development of the ELISA assay may represent a major advance in scab management, allowing for sub-clinical detection

and providing a cheaper, more rapid and more reliable method than physical mite detection, since the latter requires the presence of clinical signs to provoke veterinary investigation (Whitesides, 2006; Busin *et al.*, 2015). Perhaps the most promising development of these technologies is the transfer of lab-based ELISA to paper (P-ELISA). Whilst this method has significant potential, the sensitivity is still considerably lower than that of lab-based assays. The acute phase proteins, haptoglobin (Hp) and serum amyloid (SAA) have been identified through serological assay as conclusive biomarkers for sheep scab (Wells *et al.,* 2013). The advantage of a further refined P-ELISA with accurate in-field specificity and multi-antigen sensitivity, detecting the acute-phase proteins and scab associated antibodies such as IgG, is that it would allow for pre-clinical signs detection, accessible testing and the removal of a significant proportion of the associated costs of clinical diagnosis. Regulated periodic screening using commercially available P-ELISA testing may be able to detect sub-clinical scab in time to prevent spread (Busin *et al.*, 2015). This could lower mass exposure and the over-use of MLs which contribute to resistance development and associated selection pressures favouring resistant alleles (Bisdorff and Wall, 2008).

To counteract the spread of resistance, co-operation between farmers including co-ordination of treatment, and novel approaches to diagnosis and treatment are required (Scott *et al.,* 2007). This is likely to require the development and implementation of integrated approaches which give significant attention to maximising animal welfare and minimising resistance development. Given the lack of compliance and co-operation often evident in farming communities (Rose *et al*., 2011; Nixon *et al,.* 2017), this may require government intervention. A centrally-managed, cooperative system with consistent monitoring may be the only way to keep prevalence below levels that are commensurate with good animal welfare in the UK.

The evidence presented in the current study implies a shared target mechanism in *P. ovis* which confers resistance across all three MLs tested. This provides important knowledge when combined with recent genetic developments in sequencing a draft *P. ovis* genome and in silencing *P. ovis* transcripts (Burgess *et al.,* 2018; Marr *et al.,*

2018). Further investigation guided by the knowledge of these recent findings, is paramount in allowing for considerably more optimism in the wake of approaching uncertainties when facing a resistant generation of sheep scab.

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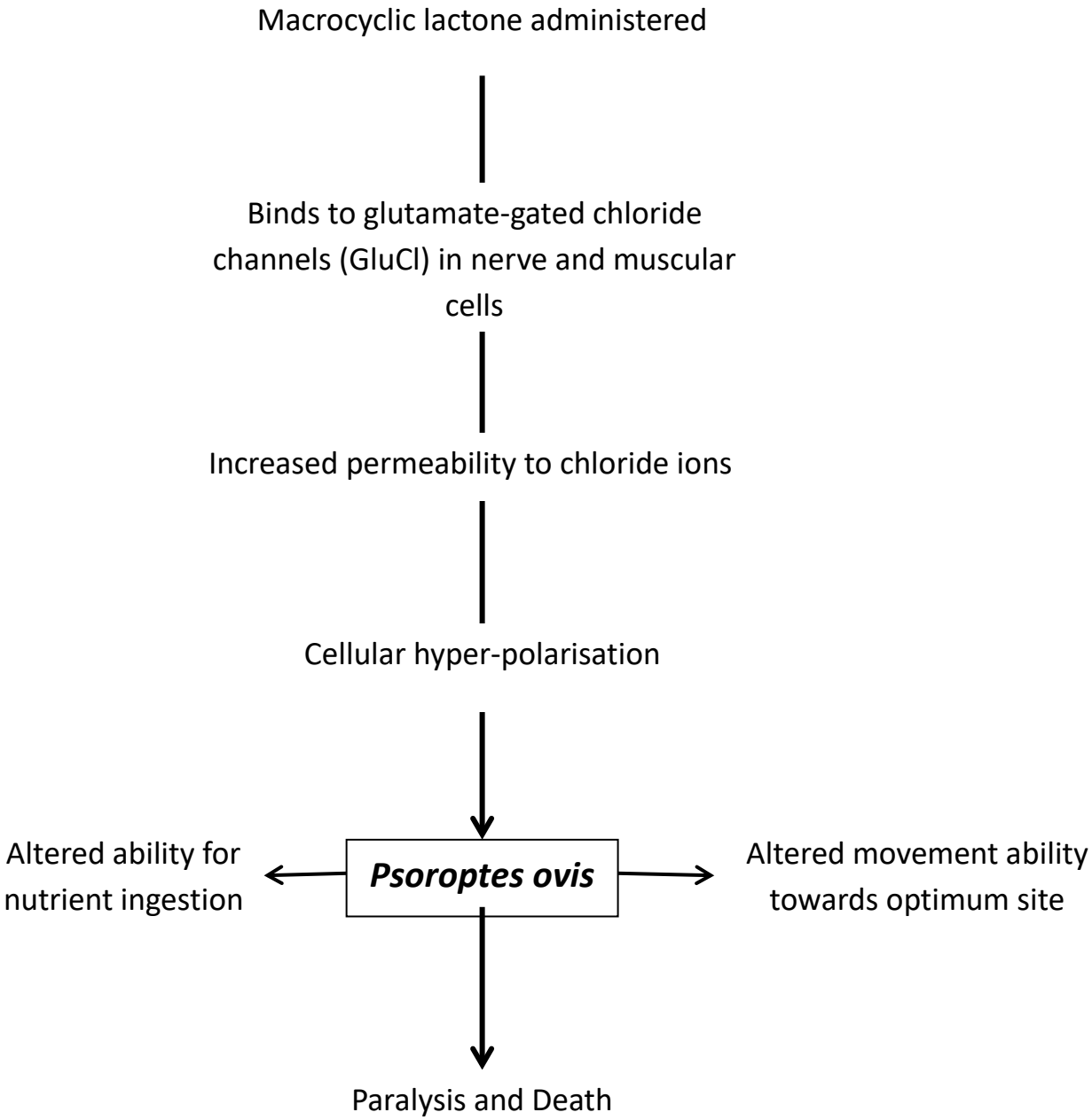
APPENDICES:

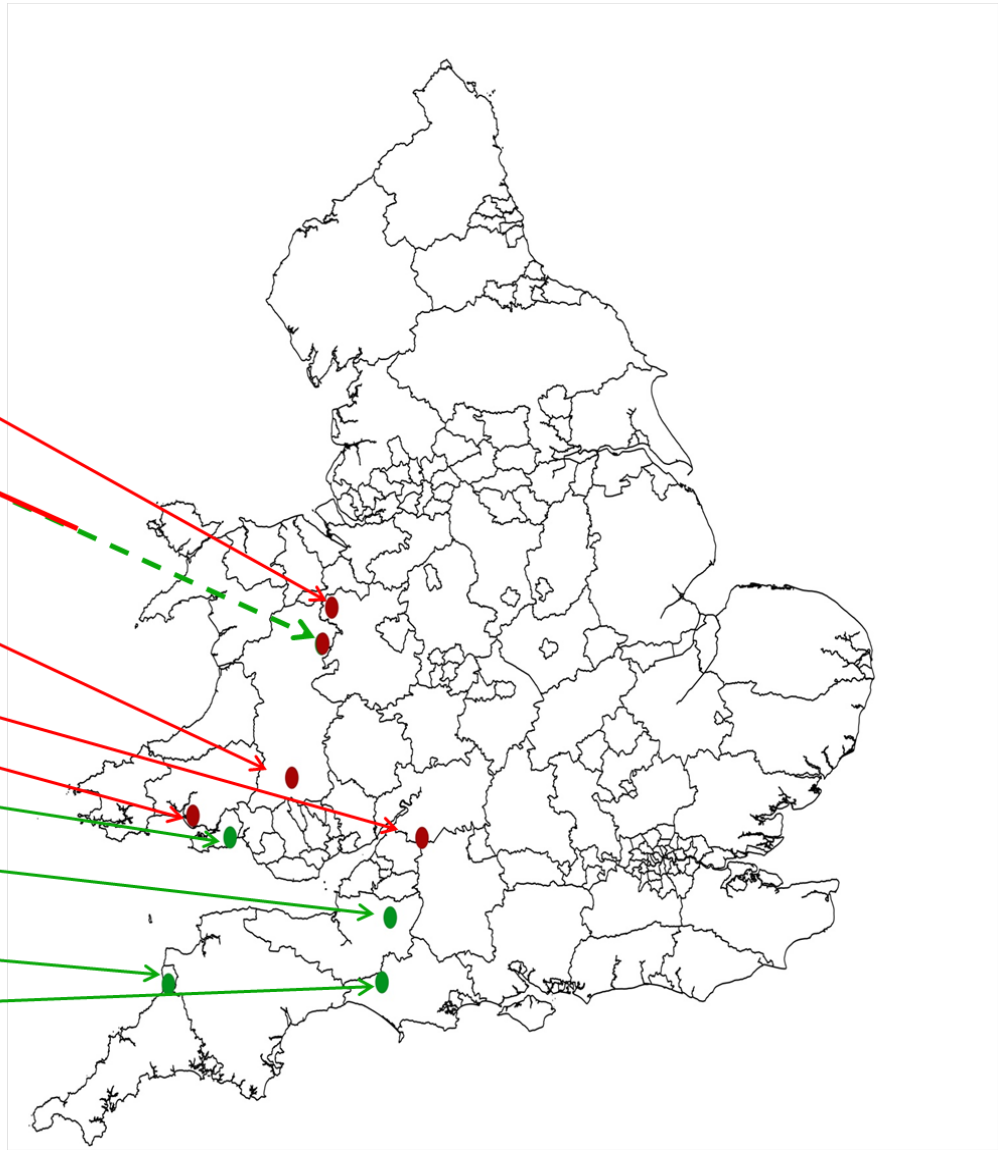


Appendix A: Schematic representation of the proposed mode of action for the macrocyclic lactone endectocides: Based on Wolstenholme and A.T. Rogers (2002).

Appendix B. A summary table of the acquisition date, origin and previous treatment history (treatment date, dose, active ingredient and mode of treatment) of the cases of sheep scab where sufficient mites were collected to be used for resistance testing.

Site Reference Number	Working Trial? Y/N	Origin Name	Drug Treatment History
1	N	Oswestry	Too few active mites present for trial
2	N	Camalas farm vets	Too few active mites present for trial
3	Y	Camalas farm vets	20mg/ml moxidectin 2% LA, ivermectin 200mcg/kg- Bimectin (1% ivermectin)
4	Y	Camalas farm vets	02/10/17 Cydectin 2% LA (moxidectin 20mg/ml) 7th & 14th 12/17 Bimectin 1% w/v (ivermectin - dosage 200mcg/kg) 03/01/18 Sheep dipped
5	N	Honddu Vets, Brecon	Too few active mites present for trial
6	N	Honddu Vets, Brecon	13/11/17- moxidectin 2% - i/m
7	N	Leigherton Farm (Bought from a farm at the Welsh Border: Monmouthshire)	23/11/17- 2.5ml Cydectin 2% (moxidectin) 5ml Monepantel (Zolvix- antihelmintic drug).
8	N	Camarthen Vets	22/11/17- 20mg/ml Cydectin LA (moxidectin) 06/12/17- Dectomax 10mg/ml - i/m (doramectin)
9	Y	St James Swansea	19/11/17 & 10/12/17 → Zermex LA (2% moxidectin)
10	Y	Shepton Farm Vets	15/01/18- Cydetin LA (moxidectin), ivermectin.
11	Y	Penbode Farm Vets	12/17- Molemec (1% ivermectin)
12	Y	Synergy Farm Health	Scab reported as not responding to doramectin and ivermectin treatments.

Working Trial?	Site Ref
N	1
N	2
Y	3
Y	4
N	5
N	6
N	7
N	8
Y	9
Y	10
Y	11
Y	12



Appendix C: *Psoroptes ovis spp.* UK field samples distribution collected for bioassay treatment response analysis

Sample locations with enough mites suitable for bioassay analysis

Sample locations without enough mites suitable for bioassay analysis

Appendix D: Letter written by the study investigator detailing instructions on collecting and sending samples of mites from non-responding outbreaks in order to maximise the likelihood of a successful bioassay analysis.

Dear Veterinary Groups/Animal Organisations,

Please find enclosed a clarification of the best method of delivering *Psoroptes ovis* samples that do not appear to respond to Macrocyclic lactones, in order for them to be trialled successfully. Please forward this letter to any farms vets who may also find it relevant.

Upon suspected resistance, please notify me directly which day you are collecting the scrape/sample and the expected arrival day (before sending or whilst in transit). This gives me time to prepare the necessary test plates before sample arrival and alongside other bioassay analyses. The process is time-sensitive to ensure mite condition upon assay trial.

Please detail any history of treatment you have for the duration that mites may have been present. E.g- If the sheep was treated at market a month before, which MLs, origin of sale etc. Please let me know which ML(s) the mites are not responding to when notifying that a sample has been sent/is being collected so I know which tests take priority and can have the appropriate treatments measured and ready beforehand.

Please send the package by special delivery for next morning/afternoon arrival as soon as possible after the scrape. This is to ensure the mites are not compromised from time spent off-host.

Please ensure a stamp/written sign of the parcel origin, ie- the veterinary group or sender name is on the envelope so that I know exactly which suspected case the mites are from as soon as it arrives.

Please place scrapes inside plastic pots (with cello tape around all sealed edges) or in freezer bags (with cello tape across edge) to ensure no escaping mites.

Deliveries sent on Sunday-Thursday Please address in the following format:

Professor Richard Wall
FAO C.S-Osborne
Bristol University
Life Sciences Building
24, Tyndall Avenue
Bristol, BS8 1TQ

If mites are being sent off on a **Friday or Saturday**, please deliver to the following address instead:

C. S-Osborne
XXXXXX XXX
XXXX, XXXX
Wiltshire
XXXX XXX
Contact details: XXX….XXXX….XXXX

Skin scrapes at the scab site are far more effective than wool- I cannot emphasise this enough in terms of successful/useable samples. *P. ovis* will only reside in wool incidentally and are always present at skin level at the site of scab/dermatitis. Unless there are <u>hundreds of highly active</u> mites, the wool will have too few mites to find before the sample becomes too desiccated during microscopy. In active infestations the mites will crawl out of the wool but this is very rare and they are predominately in the scrapes.
Ie-

Thank you all for your cooperation and assistance with the research, it is greatly appreciated,

Appendix E:
Summary table detailing all ML treated outbreak and ML treated MRI naïve samples against outbreak untreated controls and MRI untreated controls.

Treatment ML	(ML) Concentration	Group 1 samples	Group 2 samples	Significant difference between Group 1 and Group 2 % mortality? Y/N	Mann Whitney-U Statistic result
Control ethanol+ nutrition sheep serum	0	control outbreak	control naïve MRI	N	W = 48.5 P-value = 0.428854
moxidectin	M 4000	Treated outbreak	Treated naïve MRI	N	W=12.5 P-value= 0.699009
moxidectin	M 4000	Treated outbreak	Untreated outbreak	Y	W = 0 P-value = 0.000782803
moxidectin	M 4000	Treated naïve MRI	Untreated naïve MRI	Y	W = 0 P-value = 0.0497457
moxidectin	M 2000	Treated outbreak	Treated naïve MRI	N/A	Insufficient data
moxidectin	M 2000	Treated outbreak	Untreated outbreak	N/A	Insufficient data
moxidectin	M 2000	Treated naïve MRI	Untreated naïve MRI	Y	W = 0 P-value = 0.0497457
doramectin	D 4000	Treated outbreak	Treated naïve MRI	N	W=6.0 P-value=0.106583
doramectin	D 4000	Treated outbreak	Untreated outbreak	N	W = 9.5 P-value = 1.0
doramectin	D 4000	Treated naïve MRI	Untreated naïve MRI	Y	W = 0 P-value = 0.0435964
doramectin	D 2000	Treated outbreak	Treated naïve MRI	N/A	Insufficient data
doramectin	D 2000	Treated outbreak	Untreated outbreak	N/A	Insufficient data
doramectin	D 2000	Treated naïve MRI	Untreated naïve MRI	Y	W = 0 P-value = 0.0435964
ivermectin	Iv 4000	Treated outbreak	Treated naïve MRI	Y	W=15.0 P-value=0.034661
ivermectin	Iv 4000	Treated outbreak	Untreated outbreak	N	W = 18.5 P-value = 0.625094
ivermectin	Iv 4000	Treated naïve MRI	Untreated naïve MRI	Y	W = 0 P-value = 0.0497457
ivermectin	Iv 2000	Treated outbreak	Treated naïve MRI	N	W= 4.0 P-value=0.245277
ivermectin	Iv 2000	Treated outbreak	Untreated outbreak	N	W= 8.0 P-value=0.901379
ivermectin	Iv 2000	Treated naïve MRI	Untreated naïve MRI	N	W = 0 P-value = 0.105192